REMARKS

A. Status of claims

Upon entry of this amendment, claims 56, 59, 60, and 105-115 are pending. Claims 56, 105 and 107 are amended. Claims 109-115 are added. Support for these amendments can be found in the instant specification and claims as filed. No new matter is added.

Claims 1-55, 57-58, 61-104 have been cancelled for being drawn to non-elected subject matter. Applicants reserve the right to pursue the subject matter of these claims in a divisional application.

B. Rejections under 35 U.S.C. § 112.

Claims 56, 59, 60, 67-77 and 105-108 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a method of treating rheumatoid arthritis or viral chronic hepatitis with a depleting anti-CD69 antibody wherein the anti-CD69 antibody specifically binds SEQ ID NO:2, allegedly does not reasonably provide enablement for a method of treating the breadth of diseases encompassed by the instant claims with a depleting anti-CD69 antibody. Applicants disagree with this rejection. Nonetheless, in the interests of advancing prosecution, the present claims have been amended to recite methods of treating rheumatoid arthritis or viral chronic hepatitis. The examiner has acknowledged that the specification enables the presently claimed subject matter. Accordingly, withdraw of this rejection is respectfully requested.

C. Rejections under 35 U.S.C. § 103.

1. Claims 56, 59, 60, 67-69, 105, 107 and 108

Claims 56, 59, 60, 67-69, 105, 107 and 108 are rejected under 35 U.S.C. 103(a) as allegedly being unpatentable over Van der Lubbe (J Autoimmun. 1997 Feb; 10(1):87-97) in view of Marzio (Immunopharmacol Immunotoxicol. 1999 Aug; 21 (3):565-82, cited herewith), McInnes 1997 (Nat Med. 1997 Feb;3(2):189-95) and McInnes 1998 (Immunol Today. 1998 Feb; 19(2):75-9).

Van der Lubbe investigates immunogenicity and *in vivo* effects on T-cells of long-term CD4 monoclonal antibody treatment of patients with rheumatoid arthritis. As acknowledged on page 6 of the Office Action, Van der Lubbe does not teach the use of depleting anti-CD69 antibodies to treat rheumatoid arthritis. Nor does Van der Lubbe disclose a depleting anti-CD69 antibody. Further, Van der Lubbe does not teach the use of depleting anti-CD69 antibodies to treat viral chronic hepatitis.

Marzio is a review article that suggests that CD69 may be involved in the pathogenesis of certain of inflammatory diseases. However, Marzio also acknowledges that "the nature of the physiologic ligand/s for CD69 remains a mystery",[1/] and that a specific role for cells expressing CD69 in the pathogenesis of rheumatoid arthritis "has not been established."[2/] Marzio does not disclose a depleting anti-CD69 antibody. Marzio does not teach the use of depleting anti-CD69 antibodies to treat rheumatoid arthritis. Further, Marzio does not teach the use of depleting anti-CD69 antibodies to treat viral chronic hepatitis.

McInnes 1998, teaches that IL-15 can both recruit and expand CD45R0+ memory T-cells subsets in the synovial membrane, which, in the continued presence of IL-15 or via contact with macrophages, increases production of TNFα.[3/] To reduce TNFα production, McInnes 1998 suggests that IL-15 expression should be downgraded or IL-15 receptors should be targeted in order to decrease inflammation.[4/] McInnes 1998 does not suggest down regulation of CD69. McInnes 1998 does not suggest the use of a depleting anti-CD69 antibody molecule, and antibody that mediates depletion of CD69+ cells. No animal study data was shown in the teachings of McInnes 1998. Thus, McInnes 1998 does not disclose and does not enable a method of treating a subject with a depleting anti-CD69 antibody molecule of the claims.

McInnes 1997 teaches that peripheral blood T-cells and U937 cells that are co-cultured in the presence of IL-15 *in vitro* have decreased TNFα production when treated with a neutralizing antibody to CD69.[5/] There are no teachings in McInnes 1997 that these antibodies deplete the

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1/ Marzio at page 573, lines 8-9.

2/ Marzio at page 574, lines 19-20.

3/ *See* McInnes 1998 at page 77, column 1, first full paragraph.

4/ *See* McInnes 1998 at page 78, column 2, first full paragraph.

5/ *See* McInnes 1997 at page 193, Figure 7 legend.

cultures of $CD69^+$ cells. McInnes 1997 does not suggest down regulation of CD69. McInnes 1997 does not suggest the use of a depleting anti-CD69 antibody molecule. No animal study data was shown in the teachings of McInnes 1997. Thus, McInnes 1997 does not disclose and does not enable a method of treating a subject with a depleting anti-CD69 antibody molecule of the claims.

Indeed, the examiner has acknowledged that neither McInnes 1998 nor McInnes 1997 teaches the use of a depleting anti-CD69 antibody to treat rheumatoid arthritis.[6/] McInnes 1997 discloses experiments performed using a "neutralizing antibodies to CD69".[7/] These are the same experiments relied upon in the primary reference of McInnes 1998. The McInnes references, therefore, never disclose depleting anti-CD69 antibodies. Further, neither McInnes 1998 nor McInnes 1997 teach the use of depleting anti-CD69 antibodies to treat viral chronic hepatitis.

a. The Prior Art References Do Not Teach or Suggest All the Claim Limitations

The Examiner has failed to provide clear articulation of the reason(s) why the claimed invention would have been obvious. See MPEP § § 2143 and 2143.03. Applicants respectfully submit that even if, *arguendo*, one of ordinary skill in the art would have had a reasonable expectation of success to combine the cited references, the combination of reference fails to teach or suggest all of the claim limitations—that is, the references do not teach all elements of the claimed method. The primary reference of Van der Lubbe fails to disclose or suggest at least one of the elements recited in the claims — that is, Van der Lubbe fails to disclose a depleting anti-CD69 antibody. None of the secondary references disclose a depleting anti-CD69 antibody. As such, the combination of the cited references would not teach every element of the claims and therefore fails to render obvious the present claims. The references alone or in combination fail to teach or suggest every element of the claims. The Examiner fails to provide a reasonable rationale for why the missing elements would have been obvious. Accordingly, the Office Action has also failed to establish a *prima facie* case of obviousness because the cited

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6/ Final Office Action, page 5, fifth (5^{th}) full paragraph.

7/ *See* McInnes 1997 at page 193, Figure 7 legend.

references do not teach or suggest all elements of the claims. Withdrawal of this rejection is respectfully requested.

b. The Cited References Fail to Provide the Requisite Motivation to Combine

Applicants assert that there is no motivation to combine the teachings of cited references in order to arrive at the instant invention. As is explained by the Federal Circuit, the motivation to combine is part of the discussion in determining the scope and content of the prior art.[8] Thus, where all claim limitations are found in a number of references, the fact finder must determine "[w]hat the prior art teaches... and whether it motivates a combination of teachings from different references".[9] In the present case, the cited references has failed to disclose a depleting anti-CD69 antibody. Accordingly, a person of ordinary skill in the art could not have modified the teachings of the cited references to arrive at the invention of the present claims.

Applicants further submit that the Examiner has merely asserted that a skilled artisan would have been motivated to combine the above references without identifying where in the references either explicit or implicit motivation can be found to support the rejection. Thus, the Examiner has only alleged that the references can be combined to arrive at the present invention. The mere fact that references can be combined or modified, however, does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination. See M.P.E.P. § 2143.01 (III). That is, a rejection based on a *prima facie* case of obviousness is improper without a motivation to combine the references. The Office Action combines facts and attempts to provide a motivation to combine the references without identifying the source of the motivation. The desirability of the combination is not suggested in any of the references cited by examiner. Accordingly, the Office Action has also failed to establish a *prima facie* case of obviousness because the cited references do not provide a reasonable rationale to combine or modify the teachings of the references to arrive at the present invention. The rationale set forth by the examiner simplifies the facts and forces a wrong conclusion.

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[8] DyStar Textilfarben GmbH & Co. Deutschland KG v. C.H. Patrick Co., 464 F.3d 1356, 1360, 80 USPQ2d 1641, 1645 (Fed. Cir. 2006); *citing* SIBIA Neurosciences, Inc. v. Cadus Pharma. Corp., 225 F.3d 1349, 1356 (Fed. Cir. 2000).

[9] *Id.* citing In re Fulton, 391 F.3d 1195, 1199-1200 (Fed. Cir. 2004).

c. The Examiner's Rejection is Based on an Impermissible Use of the Obvious to Try Standard

The examiner is using an impermissible obvious to try standard in the rejection of the present claims over the teachings of Van der Lubbe, Marzio, McInnes 1997, and McInnes 1998. An invention is not obvious if the inventor merely would have been motivated "to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical nor any direction as to which of many possible choices is likely to be successful."[10/] "Likewise, an invention would not be deemed obvious if all that was suggested 'was to explore a new technology or general approach that seemed to be a promising field of experimentation.'"[11/] In this regard, the teachings of McInnes 1998, Ledbetter and McInnes 1997, taken together, present numerous choices to be tried but do not lead one of ordinary skill in the art to a successful result.

The Federal Circuit has stressed that an invention is obvious when there is a lack of numerous parameters to vary and the prior art gives specific guidance as to how to reasonably achieve success.[12/] The Federal Circuit has consistently applied this principle in its decisions in In re O'Farrell, Pharmastem Therapeutics, Inc. v. Viacell, Inc., and Medichem, S.A. v. Rolabo, S.L. While the court found the inventions in those cases to be obvious, the facts of In Re O'Farrell, Pharmastem, and Medichem can be easily distinguished from the instant case.

In re O'Farrell involved an appeal from a decision of the U.S. Patent and Trademark Office Board of Patent Appeals and Interferences that rejected an application for obviousness.[13/] A year before they applied for a patent, two coinventors published an article that described the method for practicing their claimed invention and provided evidence suggesting the invention would be successful. In light of the article, the Federal Circuit held that their invention was obvious within the meaning of § 103.[14/]

10/ In re O'Farrell, 853 F.2d 894, 903 (Fed. Cir. 1988); *See also*, Pharmastem Therapeutics, Inc. v. Viacell, Inc., 491 F.3d 1342, 1364 (Fed. Cir. 2007) and Medichem, S.A. v. Rolabo, S.L., 437 F.3d 1157, 1166-67 (Fed. Cir. 2006).

11/ Pharmastem, 491 F.3d at 1364, quoting In re O'Farrell, 853 F.2d at 903; *See also* Medichem,. 437 F.3d at 1166-67.

12/ *See* In re O'Farrell, 853 F.2d at 903; Pharmastem, 491 F.3d at 1364; and Medichem, 437 F.3d at 1166-67.

13/ In re O'Farrell, 873 F.2d at 894.

14/ *Id.* at 904.

The appellants in In re O'Farrell argued that at the time the article was published, there was enough unpredictability in the field of molecular biology to render their claimed method of synthesizing proteins nonobvious to one of ordinary skill in the art. [15/] In its discussion of the standard under § 103, the court noted that an invention could be "obvious to try" while ultimately remaining nonobvious. [16/] For example, an invention is nonobvious when it is discovered by "vary[ing] all parameters or try[ing] each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful." [17/] Similarly, an invention is nonobvious "where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it." [18/]

"For obviousness under § 103, all that is required is a reasonable explanation of success." [19/] In In re O'Farrell, the Federal Circuit found that the information in the prior art article provided such a reasonable expectation of success.[20/] As a result, the appellants' claimed invention was deemed obvious under § 103.

In Medichem, the Federal Circuit held that the addition of a tertiary amine to a patented reactive process was obvious in view of the prior art.[21/] Recognizing that the addition of the tertiary amine was the only significant difference between the appellee's and appellant's processes, the Federal Circuit noted that the appellee's invention was "a species within the genus of the [appellant's] invention."[22/] In Medichem, the court emphasized that "there [were] not numerous parameters to vary," nor was this a case "where the prior art [gave] merely general guidance."[23/] The guidance of the prior art in Medichem was "quite clear - namely, that . . . reactions of this kind can sometimes be optimized by adding low levels of a tertiary amine."[24/]

15/ *Id.* at 902.
16/ *Id.* at 903.
17/ *Id.*
18/ *Id.*
19/ *Id.*
20/ *Id.* at 904.
21/ Medichem, 437 F.3d at 1167.
22/ Id. at 1161.
23/ Id. at 1167.
24/ Id.

As a result, the court concluded that the addition of a tertiary amine to the reactive process "would have been obvious in view of the [appellant's] patent and the prior art."[25/]

In Pharmastem, the Federal Circuit determined that patents for an umbilical cord banking process were obvious under the obvious to try standard.[26/] In that case, the two patents in question described "a process for collecting a newborn infant's umbilical cord blood at the time of birth, testing it for suitability for later use, preserving it through cryopreservation, and infusing it into an individual . . . whose hemotopoietic stem cells have been destroyed."[27/] Relying on the existence of prior art references (scientific articles that suggested using cord blood for this purpose and those that suggested cryopreservation and storage of the cord blood as needed), the court determined that "the idea of using cryopreserved cord blood to effect hematopoietic reconstitution was not new at the time the inventors filed" the patents in question.[28/]

While acknowledging that work of Pharmastem's inventors may have advanced the state of science in the field, the Federal Circuit concluded that the inventors "merely used routine research methods to prove what was already believed to be the case."[29/] In Pharmastem, there was "not an array of possible choices" as to how to achieve the scientific objective, nor were there "problems to be solved in implementing the prior art suggestion."[30/] To the contrary, the court found that "each step of the . . . [umbilical cord collection] procedure had been spelled out in the prior art."[31/] As a result, the Federal Circuit determined that it was unreasonable for a jury to reach the conclusion that the patents were not obvious and reversed the district court's denial of JMOL on that issue.[32/]

Unlike the prior art references in Pharmastem, In re O'Farrell and Medichem, which pointed inventors to specific processes and limited the number of variables to try, the references cited by the examiner do not limit an inventor to a list of potential therapeutic targets. For example, Van der Lubbe investigates immunogenicity and *in vivo* effects on T-cells of long-term CD4 monoclonal antibody treatment of patients with rheumatoid arthritis. Thus, at best, Van der

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25/ Id.
26/ Pharmastem Therapeutics, 491 F.3d at 1367.
27/ Id. at 1347.
28/ Id. at 1360.
29/ Id. at 1363.
30/ Id.
31/ Id.
32/ Id. at 1364.

Lubbe suggests CD4 as a potential therapeutic target. Van der Lubbe does not mention CD69 as a potential target — indeed, Van der Lubbe never mentions CD69.

Marzio is a review article that suggests that CD69 may be involved in the pathogenesis of certain of inflammatory diseases. However, Marzio also acknowledges that "the nature of the physiologic ligand/s for CD69 remains a mystery",[33/] and that a specific role for cells expressing CD69 in the pathogenesis of rheumatoid arthritis "has not been established."[34/] Accordingly, Marzio does not name CD69 as a therapeutic target for rheumatoid arthritis. Rather, Marzio appears to suggest CD69 may be invoveld in the pathogenesis of inflammatory diseases and that its role needs to be identified and studied.

McInnes 1998 does not refer to CD69 as a therapeutic target; indeed, in the concluding section of McInnes 1998 under the heading *Therapeutic implications*, CD69 is not even mentioned. IL-15 is highlighted as the target. Specifically, McInnes 1998 states as follows:

> The identification of IL-15-mediated T-cell and monocyte activation in synovial membrane, apparently operating upstream from the effects of TNF-α, provides a novel target for such biological therapeutic approaches. This might be either through direct neutralization of IL-15 or by targeting IL-15 receptors, particularly IL-15Rα. Studies in animal models of arthritis are now required to address these exciting possibilities.

Thus, McInnes 1998 merely suggests IL-15 or one of the multitude of IL-15 receptors as potential therapeutic targets.

McInnes 1997 also does not identify CD69 as a therapeutic target. McInnes 1997 reports to the role of IL-15 in the induction of TNFα production in rheumatoid arthritis through activation of synovial T cells, which often express CD69, HLA-DR, and VLA1.[35/] The experimental result in McInnes 1997 shows that antibodies against CD69, LFA-1, and ICAM-1 significantly inhibited the ability of T cells to activate macrophages by cell contact, thereby implicating IL-15 as the source of the induction of TNFα production as opposed to other cytokines such as IL-2. Although CD69 is identified as a participant in the IL-15 molecular pathways, CD69 is not taught as a potential therapeutic target. Indeed, McInnes 1997 concludes

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33/ Marzio at page 573, lines 8-9.

34/ Marzio at page 574, lines 19-20.

35/ *See* McInnes 1997 at abstract and page 192, right column, last paragraph.

with a list of implications of the results that in no way discloses CD69 as a potential target; all focus is placed on IL-15. This conclusion is evidenced by the fact that McInnes 1998, a review article published a year after McInnes 1997 (see excerpt above), fails to expressly identify CD69 as a therapeutic target. Thus, even with the benefit of the data set forth in McInnes 1997, the main authors of McInnes 1997 failed to identify CD69 as a potential therapeutic target.

Furthermore, even assuming, *arguendo*, that McInnes 1998 does suggest molecules other than IL-15 as possible therapeutic targets, the list of other possible targets would have to be expanded include each of the molecules implicated by McInnes 1998 to be involved in the biological pathway that links IL15 expression to inflammation in rheumatoid arthritis.[36/]

Taken together, the references cited by the examiner, in the least, identify the following molecules to be potentially involved in the pathogenesis of rheumatoid arthritis:

1. CD4;
2. IL-15;
3. the myriad of IL-15 receptors, particularly IL15Rα;
4. TNF-α;
5. leukocyte function-associated molecule 1 (LFA-1),
6. intercellular adhesion molecule (ICAM-1), and
7. CD69.

Thus, at best, the references relied on by the examiner only provide general guidance to a promising filed of experimentation and provide insight to a biological mechanism hypothesized to be involved in the inflammation seen in rheumatoid arthritis. Unlike the prior art reference in Pharmastem, which spelled out each step of the procedure in question, the references relied on by the examiner point implicate numerous molecules as potentially involved in pathogenesis of rheumatoid arthritis.

d. Any Prima Facie Case of Obviousness is Rebutted By Evidence of Unexpected Results Set Forth in the Specification

Applicants respectfully submit that a person having ordinary skill in the art reviewing the cited references would not have had a reasonable expectation of success at arriving at the methods of the present claims, in part, because of the methods of the claims are based on unexpected results.

36/ *See e.g.*, McInnes 1998 at page 78, right column, first full paragraph and page 77, top of left column.

Appellants submit, as explained above, that the Examiner has not established a *prima facie* case of obviousness. However, even if the Examiner had presented such a case (Appellants submit that the Examiner did not present such a case), this case would be rebutted by the unexpected results presented in the instant application. As set forth in KSR, "combining elements that work together 'in an unexpected and fruitful manner' would not have been obvious."[37/]

Appellants submit that the methods of the present claims are based upon unexpected results that shows that depleting anti-CD69 antibody molecules are effective in an *in vivo* model for unwanted immune response. The specification teaches, unexpectedly from the standpoint of one of ordinary skill in the art at the time the invention was made, that it is important that the CD69 specific antibody be a depletor of CD69+ cells, as opposed to specifically binding to CD69, while not depleting CD69+ cells in an *in vivo* model for unwanted immune response. Treatment of mice having collagen-induced arthritis (CIA) with a CD69 specific antibody that does not deplete CD69+ cells *in vivo* (*i.e.*, mAb 2.2) actually exacerbated CIA in those mice.[38/]

The examiner refutes the evidence of unexpected results by alleging that there is no nexus between the present claims and the evidence demonstrating that treatment of mice having collagen-induced arthritis (CIA) with a CD69 specific antibody that does not deplete CD69+ cells *in vivo* (*i.e.*, mAb 2.2) actually exacerbated CIA in those mice. On the contrary, however, a nexus does exists. Such evidence demonstrates the criticality of the recited element — depleting anti-CD69 antibody molecules — *which is an element that is absent in each an every reference that has ever been cited by the Examiner.*

In view of the above, withdrawal of this rejection is respectfully requested.

2. Claims 105 and 106

Claims 105 and 106 are rejected under 35 U.S.C. 103(a) as unpatentable over Van der Lubbe (J Autoimmun. 1997 Feb; 10(1):87-97, cited herewith) in view of Marzio (Immunopharmacol Immunotoxicol. 1999 Aug;2 1 (3):565-82, cited herewith), McInnes 1997 (Nat Med. 1997 Feb;3(2):189-95) and McInnes 1998 (Immunol Today. 1998 Feb;19(2):75-9). as

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37/ KSR, 127 S. Ct. at 1740.

38/ *See e.g.*, Specification at page 105, lines 3-6.

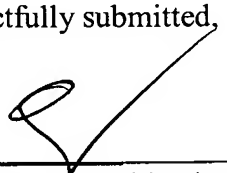
applied to claims 56,59,60, 67-69, 105, 107 and 108 above, and further in view of White (U.S. Patent Publication No. 2002/0039557 Al).

The references of Van der Lubbe, Marzio, McInnes 1997, and McInnes 1998, are discussed above. As mentioned, none of these references teach a depleting anti-CD69 antibodies, nor do these references teach the use of depleting anti-CD69 antibodies to treat rheumatoid arthritis or viral chronic hepatitis. White does not teach a depleting anti-CD69 antibody or any use thereof. White does not mentioned CD69 as a potential therapeutic target — indeed, White never mentions CD69. Rather, White is relied on by the examiner for the use of a radioisotope as a conjugated second therapeutic that may render the conjugated antibody as a depleting antibody. As such, White does not cure the deficiencies of these references, which forces the conclusion that the combination of the prior art references do not teach or suggest the elements of the claims, and therefore, fail to render obvious the present claims. As such, applicants respectfully submit that the rejection fails to establish a *prima facie* case of obviousness. Withdrawal of this rejection is respectfully requested.

CONCLUSION

On the basis of the foregoing amendments and remarks, Applicant respectfully submits that this paper is fully responsive and that the pending claims are in condition for allowance. Such action is respectfully requested. If there are any questions regarding these amendments and remarks, the Examiner is encouraged to contact the undersigned at the telephone number provided below.

Respectfully submitted,



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(54) Title: IMMUNOMODULATION USING NKR-P1, CD69 AND LIGANDS THEREFOR

(57) Abstract

Monosaccharide and oligosaccharide ligands for NKR-P1 and CD69, expressed on the surface of effector cells of the immune system, including Natural Killer (NK) cells, are identified and demonstrated to be useful in enhancing and inhibiting effector function, including cytotoxicity. Effector function is enhanced when ligands are clustered, for example on liposomes or engineered amino acid sequences, and inhibited when the ligands are in monomeric or free form. Ligands and/or effector cells may be targetted to target cells using members of specific binding pairs, such as antibodies. Soluble forms of NKR-P1 and CD69 may also be used.

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IMMUNOMODULATION USING NKR-P1, CD69 AND LIGANDS THEREFOR

The present invention relates to the modulation of components of the immune system. It is founded on the identification of ligands, especially saccharide ligands, for receptors found on the surface of cells involved in the immune response, receptors including NKR-P1 and CD69. Mono- and oligo-saccharides are shown to be able to inhibit or activate effector cells depending on whether they are monomeric (free) or clustered.

Natural killer (NK) cells are cytotoxic lymphocytes that are able to lyse a variety of target cells, including tumour cells, virus- or intracellular bacteria-infected cells (12). However, the role of NK cells in the immune system extends beyond cell-killing. NK cells have receptors for class I MHC on their surface, able to recognise both autologous and allogeneic class I alleles (63), suggesting a role in recognition of changes in conformation of class I antigens, which may take place for instance during viral infection. Class II-restricted helper T cells and antigen-presenting cells are not required by NK cells allowing likely detection of pathological changes in cells of most tissues, including haematopoeitic and endothelial cells.

Activation of NK cells results not only in cytotoxicity, but also in secretion of cytokines, particularly γ-interferon (IFN-γ),

granulocyte/macrophage colony-stimulatory factor (GM-CSF) and tumour necrosis factor (12). IFN-γ and GM-CSF are strong activators of phagocytic cells, contributing to inflammation, and activation of antigen-presenting cells. NK cells play an important role in modulating antigen-specific response of helper and cytotoxic T cells (65). Indeed, as reviewed by Trinchieri (63), the early NK cell response during infection has profound effects on the characteristics of the ensuing antigen-specific adaptive immune response, and is required for optimal response of type I helper (TH1) cells. Modulation of NK cell activity may therefore have profound effects not only in the innate immune response but also in the antigen-specific responses.

Understanding the biological roles of the diverse oligosaccharides of glycoproteins, proteoglycans and glycolipids has been a major challenge in cell biology. Work with monoclonal antibodies has shown that there are striking changes in the display of oligosaccharides at the surface of cells during stages of embryonic development and cellular differentiation, and that there are predictable changes in malignancy[1,2]. This has raised the possibility that such oligosaccharides (among which are the major blood group antigens and related sequences) may have important roles as ligands in macromolecular interactions which determine the way cells migrate or respond to various micro-environments (1,3,4).

Recently knowledge has grown on proteins which contain domains with predicted carbohydrate-binding activities based on their amino acid sequences. Prominent among these are the calcium-dependent, C-type, lectin family characterized by the presence of one or more domains that resemble the carbohydrate-recognition domain (CRD) of the hepatic receptor for asialoglycoproteins (5). Several members of this protein family are now known to bind carbohydrates (6). Notable among these are the leukocyte-to-endothelium adhesion proteins, selectins(7-9), which have crucial roles in leukocyte extravasation and recruitment in inflammation and almost certainly play a role in the blood-borne spread of tumour cells.

Proteins of the C-type lectin family have been described on natural killer (NK) cells (10,11). The C-type lectin-like proteins on NK cells are dimeric type II transmembrane proteins each containing an extracellular motif common to CRDs of known C-type lectins, a stalk region and a cytoplasmic tail containing tyrosine and serine residues that are potential phosphorylation sites, and a tetrapeptide motif which on the T-lymphocyte glycoproteins CD4 and CD8 mediates association with the protein tyrosine kinase $p56^{lck}$ (refs 13,14). To date, however, oligosaccharide binding to these lectin-like proteins has not been shown and, since the proteins have been placed in the "Group V" category, lacking Ca^{2+} binding

motifs and having quite diverse sequences, ability to bind oligosaccharide has been doubted (5).

One or the first of these proteins to have been cloned and sequenced is NKR-P1 of rat (15), several isoforms of which occur simultaneously on NK cells of the rat and mouse (16,17); related proteins occur on human NK cells (10,11). Antibodies bound to NKR-P1 on the surface of NK cells can induce antibody-dependent cytotoxicity of FcR^+ target cells (18) (via the Fc portion of the antibody - so-called "retargetted killing"), and cross-linking NKR-P1 with antibodies stimulates phosphoinositide turnover and mobilization of intracellular calcium (19). Events in the cascade leading to killing include conjugate formation with the target cells, generation of inositol trisphosphate ($InsP_3$) and inositol bisphosphate ($InsP_2$), and an increase in free cytoplasmic calcium in the killer cells prior to the release of pore-forming proteins and cytotoxic factors from their intracellular granules (12,19,20).

CD69 (Schwarting *et al* 1989; Testi *et al* 1994 and Ziegler *et al* 1994a) is a cell surface molecule, a homodimer with subunits in the range 26-34 kDa, which in vivo is constitutively expressed on activated lymphocytes such as $CD3^{bright}$ thymocytes, T cells in lymph nodes, the tonsil and the gut lamina propria, as well as blood monocytes, epidermal Langerhans cells, bone marrow myeloid cells and platelets. Otherwise the

expression of CD69 can be induced upon stimulation of most cells of hematopoietic origin. Because of the broad distribution of CD69 and its ability to generate intracellular signals, it has been suggested that the protein has a general role in the biology of hematopoietic cells, including cell activation and differentiation. It may act as a common cellular trigger (57). It has been said that the definition of CD69 ligands and requirements for their expression should uncover a molecular interaction of potential general relevance for the activation and function of haematopoietic cells (57).

Molecular cloning (Hamann *et al* 1993; Lopez-Cabrera *et al* 1993; Ziegler *et al* 1993) has revealed that CD69 is a type II transmembrane protein with a molecular mass 22, 559 Da and a carboxy-terminal region that resembles the carbohydrate-recognition domains of Ca^{2+}-dependent (C-type) animal lectins. Dimeric proteins of this type have been assigned to C-type lectin group V (5) which includes the natural killer (NK) cell-associated proteins, NKR-P1, Ly-49 and NKG2 (Chambers *et al* 1993; Yokoyama and Seaman 1993).

However, CD69 differs from these latter proteins in being shorter; it lacks a "stalk" region that intervenes between the transmembrane and the "neck" region of the lectin-like domain. Chromosomal mapping has shown that both in the murine and the human, the gene for CD69 clusters with those for C-type lectins

which are expressed predominantly on NK cells, in a region termed the NK gene complex in the murine (Yokoyama *et al* 1990). Thus, in the murine, the CD69 gene maps to the distal part of chromosome 6 linked to the NKR-P1 and Ly-49 gene families (Yokoyama *et al* 1990; Ziegler *et al* 1994b), and in the human, the gene maps to a region of chromosome 12 that is synthenic to the distal part of the murine chromosome 6. However, analysis of the CD69 gene has revealed a single copy in contrast to gene families that exist for NKR-P1 and Ly-49. (Santis *et al*, 1994; Ziegler, *et al*, 1994b).

Ligands for CD69 have not previously been identified. CD69 having been assigned to C-type lectin Group V, its ability to bind oligosaccharide has been doubted (5).

In *in vitro* experiments where CD69 on lymphocytes was cross-linked using anti-CD69, several effects were observed (if the lymphocytes were first stimulated with phorbol myrstate acetate, a stimulator of protein kinase C): (a) human T cells, B cells and thymocytes were induced to proliferate; (b) in thymocytes there was stimulation of proliferation and stimulation of the expression of genes coding for interleukin-2, interferon-γ (58, 59), and tumour necrosis factor-α (60). In other experiments evidence was obtained that CD69 on activated T cells, upon contact with monocytes, results in stimulation of interleukin-1β production in these latter cells (61, 62). Thus, it appears that the

docking of CD69 on T lymphocytes with its ligands may on the one hand stimulate proliferation and production of cytokines in the same cells, and on the other hand stimulate ligand-bearing cells to which CD69 binds, eg monocytes, to produce their own cytokines.

In platelets and circulating monocytes where CD69 expression is constitutive, cross-linking of CD69 with antibodies results in activation of cytosolic phospholipade A2. This pathway is known to lead to generation of oxidised metabolites of arachinoic acid. There is thus a link up with pathways of thromobostasis platelets (clotting mechanisms) and also allergic states (monocyte activation).

As discussed further below, we have shown that CD69 and NKR-P1 are lectins which are intimately involved in the natural killing process. The identification of ligands for CD69 and NKR-P1 has wide implications not only for modulation of cell-killing activity, but also for modulation of cytokine production, and thus anti-inflammatory and immuno-modulatory drug design.

Numerous lectin-like molecules of this family are known to occur on human lymphocytes, for example on B lymphocytes, monocytes and accessory cells (5, 11). Carbohydrate ligands, or synthetic mimicks or mimetics thereof, therefore have potential as therapeutic agents in stimulating or inhibiting natural killing, T-cell mediated cytotoxicity and other bi-directional immune

cell interactions, eg B-cell/T-cell interactions, or accessory cell/T-cell or accessory cell/B-cell interactions that are known to have important roles in the immune response.

The present invention in various aspects is based on results obtained (as disclosed herein), including the following significant findings:

1 **It is established that NKR-P1 and CD69 are directly involved in the natural killing (NK) process.**

Ryan et al (19) previously showed that aggregation of NKR-P1 on the NK cell surface using an antibody can stimulate phosphoinositite turnover and a rise in intracellular calcium, but did not establish a role for the protein in the natural killing process. The antibody failed to block both natural killing of YAC-1 target cells and phosphoinositide turnover elicited by these target cells. The authors concluded that the YAC-1 killing pathway does not involve NKR-P1. It is shown here that it does.

It is also shown herein that CD69 is expressed very quickly in NK killing, more quickly than new protein synthesis allows. NK killing is inhibitable with CD69 protein.

2 **Ligands are identified for NKR-P1 and CD69.**

Some of these are of very high affinity, with IC_{50} values ranging from 10^{-9} to 10^{-12} M.

The presence of a lectin-like domain on NKR-P1 has been described (15). Ability to bind the

monosaccharides N-acetylgalactosamine, N-acetylglucosamine and fucose (IC_{50} values 0.6×10^{-7}, 2×10^{-7} and 2×10^{-6} M respectively) is described in a paper unpublished at the first priority date claimed herein (21). The mono- and oligo-saccharides now identified have affinities which are a hundred to a hundred thousand times more.

The predicted protein sequence for CD69 is that for a lectin-like molecule (54-56) although carbohydrate-binding by this protein has not previously been reported. It is now shown herein that CD69 is a lectin and has carbohydrate-binding specificity which overlaps with that of NKR-P1. Heparin and chondroitin sulphate disaccharides (see Table 2) are high affinity ligands for both NKR-P1 and CD69. The keratan sulphate disaccharide and O-glycosidic disaccharide shown are particularly good for CD69.

Evidence that oligosaccharide structures of chondroitin sulphate type and O-glycosidic type are ligands for CD69 on NK-susceptible target cells is provided.

3 Monomeric ("free") mono- and oligo-saccharide ligands are shown to inhibit natural killing and to inhibit activation of NK cells (evidenced by increase in phosphoinositide levels and free cytoplasmic calcium).

The results herein show inhibition of target cell killing by natural killer cells. Similar results have

been obtained using ligands for NKR-P1 and ligands for CD69.

Certain of the saccharides (eg GM2 (27)) shown herein to interact with NKR-P1 have been shown previously to be present on NK-susceptible target cells, or were shown to inhibit natural killing (eg heparin (34), luquoid (53) and mannose-6-phosphate (36)). However, it was not known how these saccharides were involved. Most inhibitory substances were large molecules, exceptions being mannose-6-phosphate and other phophorylated and sulphated monosaccharides. Research on a role for mannose-6-phosphate in natural killing was previously abandoned because there were no ideas on mechanisms of action.

4 Clustered mono- and oligo-saccharide ligands are shown to activate NK cells (evidenced by increase in phosphoinositide levels and free cytoplasmic calcium).

Ligands clustered on liposomes are shown to activate NK cells. The effect is density dependent. Similar results have been obtained using ligands for NKR-P1 and ligands for CD69.

It is particularly surprising that striking negative or positive signals can be elicited depending on the mono- or oligomeric state of the oligosaccharides (i.e. whether they are free or clustered).

5 NK-resistant cells are rendered NK-susceptible by treatment with clustered carbohydrate ligands for NKR-

P1 or CD69 (demonstrated using liposomes).

The killing of otherwise resistant cells is enhanced most when the liposomes are used to pre-treat the cells before exposure to NK cells.

According to one aspect of the present invention there is provided a method of modulating activity of effector cells of the immune system, comprising contacting the cells with a monosaccharide or an oligosaccharide which comprises a glycosaminoglycan oligosaccharide a sulphated ganglioside other than sulphatide, a 6-sialyl hexose or 3-O-sulphated uronic acid.

Preferably the oligosaccharide is a keratan sulphate, a chondroitin sulphate or a heparin sulphate. Preferred oligosaccharides include K6; Chon OS, 6S, 2,6S and 2,4,6S; and Hep IVA, IIA and IS, HNK-1, 6SN, 6S2, 6SLN, S2 and SN.

Oligosaccharide which may be used in modulating activity of Natural Killer (NK) cells include those which comprises a glycosaminoglycan oligosaccharide, sulphatide, a sulphated ganglioside other than sulphatide, a 6-sialyl hexose or 3-O-sulphated uronic acid, tetramannose phosphate, pentamannose phosphate, Sialyl- or sulphated- Le^a or Le^x; HNK-1; HNK-3-5-uronic acid.

These may be selected from keratan sulphates, such as K6; S2; SN; 6ST; Chondroitin sulphates, such as

$ChonSO_3$ OS/6S, 2,6S and 2,4,6S; Heparin oligosaccharides, such as HepIVA, IIA, IS and IS2.

Other preferred ligands, functional features and moieties shared by preferred ligands and thus defining useful groups for inclusion on preferred ligands, are readily identifiable by those skilled in the art from the disclosure herein. For instance, various features are shown to be common to more than one ligand with a high affinity for NKR-P1 and/or CD69. Those skilled in the art will readily identify these from the information given herein and will employ them in designing or choosing further ligands which may be used in accordance with the present invention.

Preferred features of monosaccharides and oligosaccharides include: α2-3 linked sialic acid, 3-O-sulphation, 3-O-sulphated galactose, 3-, 4- or 6- O-sulphated N-acetyl hexosamine, N-sulphated hexosamine, uronic acid-substituted N-acetylhexosamine or hexosamine, 2- or 3- O-sulphated uronic acid, α1-3-linked fucose, α1-4-linked fucose, α2-6-linked sialic acid, α2-6-linked sialic acid on galactose or on N-acetylhexose.

As discussed further herein, an oligosaccharide is a short chain of saccharides, as opposed to a polysaccharide. Preferred oligosaccharides are di-, tetra-, penta-, hexa- and hepta- saccharides, and oligosaccharides of greater length, though preferably less than 25 residues.

As discussed, "free" or monomeric, i.e. unclustered ligands may be used to inhibit effector function, whereas clustered ligands may be used to augment, enhance or increase function.

Cells at which effector function is directed ("target cells") may be treated with the monosaccharide or oligosaccharide and then treated with the effector cells, or treated first with the effector cells then monosaccharide or oligosaccharide. The treatment with monosaccharide or oligosaccharide, or the treatment with effector cells may be targetted to the target cells, for example using a member of a specific binding pair such as an antibody or antibody fragment able to bind to an antigen, such as an antigen on the surface of target cells.

As discussed, clustering may be on a liposome, and the liposome may comprise a first member of a specific binding pair (sbp member) able to bind a complementary second member of the specific binding pair (e.g. antibody or antibody fragment).

Alternatively, clustering may be on a sequence of amino acids, as discussed, which may be part of a molecule such as an antibody or other specific binding pair member.

The activity modulated may be anti-proliferative activity, cytotoxicity and/or cytokine secretion, or any other effector function of a cell of the immune system. Cytotoxicity may comprise an apoptotic

element, as discussed, particularly when the ligand binds CD69.

Tumour cells are a preferred target, as are with virally infected cells.

Treatment may be *in vitro* or *in vivo*.

Further aspects of the present invention provide ligands as disclosed in here for use in the manufacture of compositions or medicaments for treatments as disclosed, and such compositions and medicaments for use in such treatments.

A further aspect of the present invention provides a method for obtaining a ligand of NKR-P1 and/or CD69, comprising screening molecules for ability to bind NKR-P1 and/or CD69, or a fragment (e.g. a soluble fragment) of NKR-P1 and/or CD69, and selecting a molecule which has said ability. Selection may be using any method available to the person skilled in the art, including those disclosed herein, and may be direct or indirect, e.g. involve competition between test molecules and a known ligand for CD69 and/or NKR-P1.

The NKR-P1 and/or CD69, or fragment of NKR-P1 and/or CD69, may be labelled.

A ligand obtained/selected in accordance with this aspect of the present invention may be a monosaccharide or oligosaccharide, and the monosaccharide or oligosaccharide may be part of a glycopeptide, glycoprotein, glycolipid or proteoglycan.

Following selection of a ligand of NKR-P1 and/or CD69 using a method according to the present invention, the ligand may be used in modulation of activity of effector cells of the immune system, such as NK cells, in a method comprising contacting the cells with the ligand, in accordance with any of the methods disclosed herein or otherwise available to those skilled in the art.

Aspects of the present invention provide methods of modulation of components of the immune system, including stimulation and inhibition of effector cell activity, such as the cell-killing activity (cytotoxicity) (or other activity inhibiting proliferation of target cells) or cytokine-secretion of NK cells or other cells. Ligands identified herein, including mono- and oligo-saccharides, may be used in methods of treatment of mammals and in compositions for use in such methods and may be used in the manufacture of medicaments for use in such method. It is likely that immunomodulatory effects of mono- and oligo-saccharides can be obtained using synthetic mimicking molecules, perhaps small and negatively charged. Such mimicks or mimetics are included within the scope of the present invention.

An alternative way of expressing the present invention focuses on target cells and the use of ligands as disclosed herein to mark them as targets for effector function of effector cells, e.g. distruction.

Treatment of target cells with a ligand of NKR-P1 and/or CD69 may increase or decrease (according to various embodiments of the present invention) their susceptibility or resistance to the effector function, e.g. anti-proliferative action, cytokine secretion, cytotoxicity. Such treament may or may not be targetted in any of the manners contemplated herein.

Soluble forms of NKR-P1 and/or CD69 may be used in immunomodulation in accordance with further aspects of the present invention, including methods of treatment of the human or animal body, compositions for such treatment and in the manufacture of compositions or medicaments for such treatment, as well as *in vitro* methods, essentially as disclosed herein for various embodiments employing mono- or oligo-saccharides. Soluble NKR-P1 and/or CD69 may reduce or inhibit effector function of cells of the immune system, such as NK cells. Binding of the soluble proteins to ligands, such as free or clustered oligosaccharide in the medium or on the surface of cells, may reduce the amount of ligand able to bind NKR-P1 and/or CD69 on the surface of effector cells, and so effector function may be reduced.

Compositions and medicaments comprising soluble NKR-P1 and/or CD69 fragments and made or used in accordance with various aspects and embodiments of the present invention, may be administered and/or employed in principle in any of the ways and for any of the

purposes disclosed herein: for example in liposomes, targetted using members of specific binding pairs such as antibodies and antibody fragments, and so on.

Immunomodulation according to the present invention may be used in the treatment of tumours in cancers such as solid tumours and leukaemia, eg the killing of leukaemic cells in autologous bone marrow transplants prior to transplantation, treatment of haematological (haemopoietic) disorders, eg leukopaenia such as aplastic anaemia where NK cells may be over-active in bone marrow, treatment of allergic states, particularly autoimmune diseases such as rheumatoid arthritis, treatment of parasitic infections, treatment of biological material, eg tissue or blood, containing virally-infected cells which may be targeted for NK-mediated killing, and in prevention or amelioration of graft-versus-host disease (GVH), eg by treatment of graft tissue prior to transplantation in order to lower graft NK cell activity. Other treatments wherein immunomodulation, in particular activation or inhibition of effector cell function, such as NK cell cytotoxicity, may be of benefit will be apparent to those skilled in the art.

Disease applications of the present invention may include: (1) - employing inhibition of natural killing - graft-versus-host disease, connective tissue diseases e.g. cartilage loss, arthritis, skin diseases, dyshaematopoietic diseases; (2) - employing inhibition

of apoptotic killing - ischemia reperfusion pathologies, for example associated with coronary heart disease, cardiac surgery, peripheral vascular injury after trauma, thrombotic diseases/hypercoagulable states e.g. in leukaemias or post trauma; epithelial diseases e.g. skin diseases associated with cell distruction, pemphigus/pemphigoid, other bullous skin lesions; (3) - employing inhibition of cytokine cascades in innate immune responses - including sepsis, rheumatoid arthritis, pulmonary fibrosis, glomerulonephritis; (4) - employing inhibition of cytokine cascades that result in antige-specific responses involving interactions of any of monocytes, antigen-presenting cells, B-lymphocytes, T-lymphocytes, NK cells - including allergic states such as asthma and eczema and other inflammatory diseases; autoimmunity; graft rejection; hyperreactive states during treatment of parasitic diseases and TB; (5) - involving inhibition of cytokine cascades involving epithelial cells and inflammatory cells, including inflammatory bowel disease, Crohn's disease, ulcerative cholitis, psoriasis and glomerulonephritis; (6) - employing conferring of NK-susceptibility on target cells, including precancer, cancer, solid tumours and leukaemias, viral infections/carrier states, parasitic infections.

As shown herein, NK cell cytotoxicity involves stimulation of apoptosis in target cells. The

potential benefit of making use of apoptotic ("programmed") cell death in therapy, e.g. of cancer, has been well-recognised. See, for example, Levitzky. *Eur. J. Biochem.* **226**:1-13 (1994), which recognises that many cells in tumours are quiescent and undividing. Such cells may escape chemotherapy aimed at proliferating cells, and then may begin dividing in an intermission in treatment. Apoptosis-inducing stimuli can actually kill non-dividing cells by inducing them to die. The ability to increase NK cell-mediated cytotoxicty involving apoptosis with ligands as disclosed herein may thus be particularly useful in cancer therapy (although clinically it might be best employed in combination with other anti-tumour therapies).

On the other hand, apoptosis has been indicated as a side-effect of reperfusion injury, especially reperfusion to limit ischaemic necrosis e.g. of myocardium, following stroke or in the liver. See Gottlieb et al., *The Journal of Clinical Investigation* **94**: 1621-1628, (1994). Thus, the ability to inhibit NK cell-mediated cytotoxicity involving apoptosis may be useful in reducing tissue damage as a result of reperfusion, and in other situations.

Where NK or other effector cells are over-active, an inhibitory composition comprising a mono- or oligo-saccharide ligand may be used. On the other hand, clustering of ligands may be used in other conditions

in order to increase NK or other effector cell function, eg to kill tumour cells. Clustering may be on liposomes, on repeat and/or branched sequences preferentially glycosylated within cells, or using any other suitable technique apparent to those skilled in the art. This is discussed further *infra*.

Of the mono- and oligo-saccharides which are identified herein as ligands for NKR-P1 and/or CD69, some have never been previously shown to have any effect likely to be of medical benefit, including keratan and chondroitin sulphates. These are thus provided by an aspect of the present invention for use in a method of treatment of the human or animal body by therapy. Others may have been shown previously to have or be likely to have some medical indication. However, the present invention provides mono- and oligo-saccharides and mimicks thereof for novel purposes, namely in the manufacture of medicaments for treatment of various conditions (exemplified above) by modulation of effector cell function via NKR-P1 and/or CD69. In preferred embodiments, NK effector function is modulated, ie stimulated (increased) or inhibited (reduced). NK effector function may be cytotoxicity and/or cytokine production.

In general, mono- and oligo-saccharides employed in the present invention comprise (or have) the following structure:

A(v,w)-B(1,x)-C(1-y)--D

wherein:

A is selected from the group consisting of hydrogen; a hexose that may be galactose or mannose and may be substituted with one or more charged moieties; substituted sialic acid; an aliphatic chain with one or more branched moieties; saturated or unsaturated uronic acid that may be substituted with one or more charged moieties; N-acetylglucosamine that may be substituted with one or more charged moieties; and N-acetylgalactosamine that may be substituted with one or more charged moieties;

B may be absent or selected from the group consisting of uronic acid that may be substituted with one or more charged moieties; galactose; N-acetylglucosamine that may be substituted with one or more charged moieties, galactose or fucose; N-acetylgalactosamine that may be substituted with one or more charged moieties; an aliphatic chain that may be substituted with one or more charged moieties; and an oligosaccharide chain or 3 or 4 mannose residues;

C may be absent or selected from the group consisting of uronic acid that may be substituted with one or more charged moieties; galactose; glucose; N-acetylglucosamine that may be substituted with one or more charged moieties; N-acetylgalactosamine that may be substituted with one or more charged moieties; and an aliphatic chain that may be substituted with one or more charged moieties;

D may be absent or selected from the group consisting of one or more repeats of B and/or C; remaining structural components of N-linked bi-, tri- or tetra-antennary oligosaccharides; O-glycosidic oligosaccharides and glycosaminoglycans; and other sequences serving to support or present the ligand;

the parenthetical lower case letters represent the position of bonded carbons of the indicated carbohydrates where v = 1 or 2, w = 2, 3, 4 or 6, x = 2, 3 or 4, y = 2, 3, 4 or 6.

when A is a substituted sialic acid the substitutions may be N-acetyl or O-acetyl;

when B or C is a substituted aliphatic chain, the substitutions may be selected from hydroxyl(s), acetyl amino ($NH.COCH_3$) and charged moiety(ies); and

charged moieties may be selected from the group consisting of sulphate, phosphate and carboxylic, e.g. sialic, acid (other charged groups may be employed).

Preferred mono- and oligo-saccharides for immunomodulation via binding to NKR-P1 are shown in Table 1, and identified and discussed further herein. Preferred mono- and oligo-saccharides for immunomodulation via binding to CD69 are shown in Table 2 and identified and discussed further herein.

The mono- and oligo-saccharides may be isolated by glycosidase digestion of natural glycosaminoglycans, for example using heparinases or chondroitinases, or by chemical release from mucin type glycoproteins such as

bovine submaxillary mucin, or they may be chemically synthesised as the complete sequences or their fragments or simpler (minimal) analogues displaying the desired charged groups or the minimum recognition elements.

For inhibition of effector function, the mono- or oligo-saccharide will generally be in "monomeric" or "free" form; i.e. not part of a molecule displaying or carrying another mono- or oligo-saccharide, or at least not a mono- or oligo-saccharide able to increase or augmeent effector cell activity.

On the other hand, for increasing or augmenting activity, mono- or oligo-saccharides will generally be "clustered", such that a plurality of mono- or oligo-saccharide molecules are presented to the effector cells by a vehicle.

Clustering may be achieved in a number of ways. For instance, mono- or oligo-saccharides may be clustered on the surface of liposomes. The composition and formulation of the carrier lipids may vary for optimum uptake by target cells of interest. Those skilled in the art are well able to select the best or a suitable liposome formulation for their purposes. For *in vivo* treatment, it may be desirable to take a biopsy of target tissue (e.g. a tumour) for *in vitro* analysis and determination of a suitable lipsome formulation.

As an alternative to clustering on liposomes, a

polypeptide may be used comprising an amino acid sequence which is glycosylated by host cells on recombinant expression of the polypeptide. Any mammalian cell may be used as a host cell providing the host cell can perform the necessary glycosylation to produce glycoprotein. Hepatic cell lines are particularly useful in general, as are CHO cells.

For example, the use of clustered lysines enables conjugation by reductive amination of oligosaccharide ligands with the display in the clustered state. A mucin-type amino acid sequence, eg clustered serines or threonines, may be used and modified *in vitro* or glycosylated within cultured cell lines expressing the appropriate enzymes: GalNAc transferase and sialyltransferases or sulphotransferases. Alternatively, amino acid sequences such as the consensus sequences Ser-Gly or Ser-Ala for decoration with glycosaminoglycans may be used (22, 64 and 67). By default, chondroitin sulphate chains are synthesised by cells. Flanking sequences comprising hydrophobic domains favour the biosynthesis of heparin/heparan sulphate type chains (67).

Polypeptides may, on the other hand, be glycosylated *in vitro* using glycosyltransferases.

Note that, herein, the terms "monosaccharide" and "oligosaccharide" encompass corresponding neoglycolipids, for example created by opening sugar ring then joining it to an amino lipid by reductive

amination, as discussed further below.

The term "oligosaccharide" includes disaccharide (which may be preferred for certain purposes), tetrasaccharide (again which are preferred in certain embodiments), pentasaccharide, hexasaccharide, heptasaccharide, octasaccharide and oligosaccharides or greater length, but preferably under about 50 residues, more preferably under about 30 residues, even more preferably under about 20 residues and most preferably under about 15 residues. Evidence is included herein to show that, in certain circumstances activity may increase with increasing numbers of saccharides (e.g. on increase in length from disaccharide to tetrasaccharide).

In accordance with the present invention, ligands may be administered to individuals. Administration is preferably in a "therapeutically effective amount", this being sufficient to show benefit to a patient. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, eg decisions on dosage etc, is within the responsibility of general practioners and other medical doctors.

Ligands may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Pre-treatment of cells with clustered ligand may enhance NK cell killing, even of normally NK-resistant cells.

Pharmaceutical compositions or medicaments according to the present invention, and for use in methods in accordance with the present invention, may comprise, in addition to ligand, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions or medicaments for oral administration may be in tablet, capsule, powder or liquid form. A tablet may comprise a solid carrier such as gelatin. Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For injection, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity

and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

For targeting of effect to, eg, tumour cells such as in leukaemia, virally-infected cells etc., antibodies, antibody fragments or other binding substances may be administered prior to or, preferably, simultaneously with administration of ligand. Antibody administration is routine in the art and may generally by any of the routes discussed above for ligand administration. Intravenous administration is preferred, eg in NaCl.

One way of targeting involves use of a bispecific antibody or other binding substance able to bind both target cells and the effector cells, eg tumour cells and NK cells. Thus, inactive NK cells may be brought to the tumour site prior to activation by administration of mono- or oligo-saccharide. CD56 (Leu19 antigen) is a pan-NK cell marker (66). This antigen is lacking on red cells, granulocytes, monocytes and a subset of T lymphocytes, B cells, thymocytes and platelets, though it is present on some neural tissues and tumours. Anti-CD56 specificity may therefore be used in the targeting of NK cells. A bi-

or multi-valent antibody may be used to take advantage of the dual specificity of anti-CD56 for NK cells and some tumours in targeting of NK cells to those tumours.

Another way of targeting employs a binding substance such as an antibody (which need only be mono-specific) linked to a liposome or other ligand-bearing vehicle, eg polypeptide comprising a glycosylation sequence, repeated for clustering of mono- or oligo-saccharide as discussed. Such a polypeptide may be linked to antibody (for example) as a fusion protein, created by recombinant expression from a gene fusion. If the specificity of the antibody is for target cells, eg tumour cells, the vehicle and target are brought together. The ligand on the vehicle triggers or enhances activity of circulating NK cells in the vicinity of the tumour.

One way of linking a binding substance to a ligand (e.g. mono- or oligos-accharide)-bearing vehicle employs a pair of intermediary specific binding molecules, such that the binding substance used in targetting is linked to the ligand-bearing vehicle indirectly, when the intermediary specific binding molecules bind each other. Thus, an antibody or antibody fragment, for example, may be joined (e.g. by a peptide bond as a result of expression of a fusion polypeptide from a chimaeric encoding sequence) to a first intermediary specific binding molecule, while the ligand-bearing vehicle is joined (again possibly by a

peptide bond, if the vehicle is a peptide or polypeptide) to a second, complementary intermediary specific binding molecule able to bind to the first.

Intermediary specific binding pairs may be selected for instance from pairs known for use in tagging polypeptides, including: a protein domain which forms a complex with a second (macro)molecule such as glutathione-S-transferase (Smith and Johnson, (1988) *Gene* **67**, 31-40), bovine pancreatic trypsin inhibitor, BPTI (Borijin and Nathans, (1993) *PNAS USA* **90**, 337-341); maltose binding protein, MBP (Bedouelle and Duplay (1988), *Eur. J. Biochem.* **171**, 541-549; Maina et al. (1988) *Gene* **74**, 365-373); a polypeptide sequence that can be biotinylated and thus made to interact with avidin or streptavidin (Schatz (1993) *Bio/Technology* **11**, 1138-1143); or calmodulin and a molecule able to bind calmodulin, such as mastoparan, calmidazolium or melatonin (though melatonin and calmodulin have a low half-life of dissociation); or Troponin C and molecules able to bind it. Some of these, or other possibilities, may be toxic and/or immunogenic *in vivo*, and therefore less preferred for administration to individuals, even though they may be satisfactory for use *in vitro*.

Calmodulin and Troponin C are calcium-dependent binding proteins and PCT/GB94/02420 discloses fusion of these molecules to binding substances such as antibodies and antibody fragments. These fusions may

be generated by expression from a chimaeric gene constructed using standard techniques known in the art. Both molecules in the fusion, the calcium-dependent binding protein and the specific binding substance such as an antibody, retain the ability to bind their respective complementary binding pair members. Likewise, PCT/GB94/02420 discloses joining of a molecule able to bind to a calcium-dependent binding protein (e.g. mastoparan able to bind to calmodulin) to another molecule, e.g. a polypeptide such as an antibody or antibody fragment.

Thus, an antibody with binding specificity enabling targetting to cells of choice (e.g. an antibody able to bind to an antigen expressed on the surface of cells of a tumour), may in accordance with a preferred embodiment of the present invention be joined (for instance as a fusion protein) to calmodulin (for example). A peptide able to bind calmodulin under appropriate conditions, e.g. mastoparan, may be joined to a sequence of amino acids (again for instance as a fusion protein) which carry clustered ligands for use in the present invention, for example for immunomodulation of NK cells. An antibody that binds a target cell (e.g. a tumour) may be linked to the vehicle carrying ligand (e.g. a sequence of amino acids bearing clustered mono- or oligo-saccharide) via the intermediary binding of the calmodulin and the peptide able to bind calmodulin (e.g. mastoparan). As

discussed, this format may be reversed with the antibody or other specific binding substance used for targetting being joined to a peptide such as mastoparan and the other binding molecule (such as calmodulin) being joined to the ligand-bearing vehicle, e.g. polypeptide.

The ligand-bearing vehicle in the situation where intermediary binding substances are used, may be a liposome as discussed, with one of the binding substances being joined to the liposome in accordance with known techniques. For example, a liposome bearing mono- or oligo-saccharide may incorporate a calmodulin binding ligand, such as mastoparan, enabling it to be linked to a specific binding substance such as an antibody, via calmodulin, for targetting.

An advantage of using a "double ligand" system involving an intermediary binding pair is the ready substitution of one targetting specific binding substance for another. The vehicle bearing the ligand (e.g. clustered mono- or oligo-saccharide) joined to one member of the intermediary binding pair may be used with any of an extremely large number of binding molecules for targetting and linked thereto via binding of the intermediary binding pair. Phage display technology (see e.g. WO92/01047) enables selection of antibodies and antibody fragments able to bind any antigen of interest. Selected antibodies or fragments may be cloned as fusions with calmodulin (for example)

and then employed in conjuction with ready-prepared ligand-bearing vehicles joined to a calmodulin-binding molecule such as mastoparan.

A further option along these lines involves use of a bispecific antibody, able to bind to target cells and to a vehicle for clustered mono- or oligo-saccharides, eg a polypeptide either glycosylated as discussed or linked to a liposome which bears mono- or oligo-saccharide. One arm of the bispecific antibody may be able to bind an antigen present on the surface of target (e.g. tumour cells), while the other might be able to bind to a peptide tag which may be linked to a ligand-bearing vehicle, such as a sequence of amino acids, or a liposome. A variety of tags which may be expressed with other polypeptides as fusion proteins have been used to date for recombinant proteins, including: the *myc* tag, (Munro, S., and Pelham, H.R.B. (1986) *Cell*, **46**, 291-300; Ward, E.S., Güssow, D., Griffiths, A.D., Jones, P.T. and Winter, G. (1989) *Nature*, **341**, 544-546); the Flag-peptide (Hopp, T.P. et al (1988) *BioTechnology*, **6**, 1204-1210); the KT3 epitope (Martin, G.A. et al (1990) *Cell*, **63**, 843-849; Martin, G.A.,et al (1992) *Science*, **255**, 192-194); an α-tubulin epitope (Skinner, R.H. et al (1991) *J.Biol.Chem.*, **266**, 14163-14166); the T7 gene 10 protein peptide tag (Lutz-Freyermuth, C., Query, C.C. and Keene, J.D. (1990) *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 6393-6397).

Polypeptides, including antibodies, may be linked

to liposomes using a hydrophobic membrane-spanning amino acid sequence (e.g. from MHC-I) or via a (glyco) phospholipid anchor domain from a protein such as Decay Accelerating Factor (mDAF - see WO89/01041, for example). A consensus amino acid sequence for phosphoinositol linkage to lipid, eg in a liposome, is available. Those skilled in the art are well versed with these techniques.

It should be noted that other specific binding pairs may be employed instead of antibody/antigen, where available. For example, peptide ligands (e.g. small hormones, neuropeptides) of cell-surface receptors may be used to mark cells bearing those receptors for destruction. For instance, the peptide ligand may be joined to a liposome bearing mono- or oligo-saccharides or joined, e.g. via a peptide bond following expression as a fusion protein, to a glycosylated sequence of amino acids (i.e. bearing clustered mono- or oligo-saccharide).

Antibodies which are specific for a target of interest may be obtained using techniques which are standard in the art. Methods of producing antibodies include immunising a mammal (eg mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. For

instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al, 1992, Nature 357: 80-82).

The production of monoclonal antibodies is well established in the art. Monoclonal antibodies can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP184187A, GB 2188638A or EP-A-0239400. One reason for doing this might be to "humanise" a non-human antibody to increase half-life upon administration to a human.

As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, eg using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with any of the proteins (or fragments), or may be one constructed using

sequences obtained from an organism which has been exposed to the antigen of interest.

It may be desirable to "humanise" non-human (eg murine) antibodies to provide antibodies having the antigen binding properties of the non-human antibody, while minimising the immunogenic response of the antibodies, eg when they are used in human therapy. Thus, humanised antibodies may comprise framework regions derived from human immunoglobulins (acceptor antibody) in which residues from one or more complementary determining regions (CDR's) are replaced by residues from CDR's of a non-human species (donor antibody) such as mouse, rat or rabbit antibody having the desired properties, eg specificity, affinity or capacity. Some of the framework residues of the human antibody may also be replaced by corresponding non-human residues, or by residues not present in either donor or acceptor antibodies. These modifications are made to the further refine and optimise the properties of the antibody.

Phage display technology also provides a means of "humanising" a non-human antibody, which may be preferred for administration to a human. Two-stages of "chain shuffling" replace non-human VL and VH domains which cooperate to form a binding site for the antigen of interest with human VL and VH domains able to bind the same antigen (PCT/GB92/01755). Nucleic acid encoding these human VL and VH domains may be fused to

sequences encoding human constant domains for expression of completely human whole antibodies.

Antibodies may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus this covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or synthetic. Chimaeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimaeric antibodies are described in EP-A-0120694 and EP-A-0125023.

It has been shown that the function of binding antigens can be performed by fragments of a whole antibody. Example binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')2 fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site; (viii)

bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al Proc. Natl. Acad. Sci. USA 90 6444-6448, 1993).

Diabodies are multimers of polypeptides, each polypeptide comprising a first domain comprising a binding region of an immunoglobulin light chain and a second domain comprising a binding region of an immunoglobulin heavy chain, the two domains being linked (eg by a peptide linker) but unable to associate with each other to form an antigen binding site: antigen binding sites are formed by the association of the first domain of one polypeptide within the multimer with the second domain of another polypeptide within the multimer (WO94/13804).

Where bispecific antibodies are to be used, these may be conventional bispecific antibodies, which can be manufactured in a variety of ways (Holliger, P. and Winter G. Current Opinion Biotechnol. 4, 446-449 (1993)), eg prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned in the preceding paragraph. It may be preferable to use scFv dimers or diabodies rather than whole antibodies. Diabodies and scFv can be constructed without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction. Other forms of bispecific

antibodies include the single chain "Janusins" described in Traunecker et al, Embo Journal, 10, 3655-3659, (1991).

Bispecific diabodies, as opposed to bispecific whole antibodies, are also particularly useful because they can be readily constructed and expressed in *E.coli*. Diabodies (and many other polypeptides such as antibody fragments) of appropriate binding specificities can be readily selected using phage display (WO94/13804) from libraries. If one arm of the diabody is to be kept constant, for instance, with a specificity directed against an NK cell-surface antigen, then a library can be made where the other arm is varied and an antibody of appropriate specificity selected.

Thus, using tumour killing as an example, one antigen binding site may be directed against a tumour marker while the other may be directed against an antigen present on an effector cell-type, eg NK cell. Inactive NK cells and target cells may then be brought together prior to triggering of NK cell activity. Previously, bispecific antibodies incorporating a specificity for the T-cell co-receptor CD3 have been shown to inhibit tumour growth (Titus, J. A. et al., J. Immunol. **138**, 4018-4022 (1987)) and to cure lymphoma (Brissinck J. et al, J. Immunol. **174**, 4019-4026 (1991)).

Alternatives and variations of all modes of

implementation of various aspects of the present invention will be apparent to those skilled in the art. Techniques are suggested by way of example and not limitation and may be modified, or alternatives employed, according to the knowledge of those skilled in the art, without departure from the scope of the present invention.

All documents mentioned in the text are hereby incorporated by reference.

Figure 1 shows binding of radioiodinated NKR-P1 protein to lipid-linked oligosaccharides (for which sequences and abbreviations are given in Table 1).

Figure 1a shows the results of chromatography of lipid-linked oligosaccharides, either stained chemically with orcinol (negative images shown in lanes 1 to 5) or overlaid with ^{125}I-labelled sNKR-P1 and autoradiographed (lanes 1′ to 5′). Lanes 1 and 1′ contained LNT and H5; lanes 2 and 2′ LNNT and LX5; lanes 3 and 3′, lactose (LAC), DLNN, LNT, LA5 and LB6; lanes 4 and 4′, 6SL; lanes 5 and 5′, 3SL; chromatography was upward.

Figure 1b shows ^{125}I-labelled sNKR-P1 protein binding (x 10^{-4} cpm) to lipid-linked oligosaccharides immobilised on plastic microwells in the presence of the carrier lipids cholesterol and egg lecithin. In the main panel, the ascending parts of the binding curves are shown for the serial dilutions of selected

lipid-linked oligosaccharides applied onto the wells, and overlaid with ^{125}I-sNKR-P1, 3 x 10^5 cpm per well. In the inset, to demonstrate saturability of binding, ^{125}I-labelled sNKR-P1 was mixed with unlabelled protein to give 10^6 cpm/μg, and applied at increasing levels to wells coated with the lipid-linked oligosaccharides GM2 and DLNN (2 pmol applied per well). Symbols: □ , LNNT; ■ , LNT; ○ , DLNN; ▼ , GA2; ●, GM2; Δ , SUA5; ▲ , IS.

Figures 1c and 1d show the intensities of binding to lipid-linked oligosaccharides, c at coating levels of 64 pmol per well and d at 8 pmol per well.

Figure 2 shows inhibition of the binding of radioiodinated sNKR-P1 to immobilized DLNN by free oligosaccharides and polysaccharides.

Figures 2a and 2c show % inhibition of the binding of ^{125}I-sNKR-P1 and DLNN in the presence of the indicated concentrations of saccharides (M or μg/ml).

Figure 2b shows the correlation between the activities of several oligosaccharides examined in both binding and inhibition assays.

Figure 3 shows comparisons of the potencies of oligosaccharides as inhibitors of the binding of radioiodinated sNKR-P1 to different oligosaccharide coats; and comparisons of the potencies of these oligosaccharides and those of unlabelled sNKR-P1 and chondroitin sulphate A (abbreviation CA) as inhibitors of the binding of ^{125}I-sNKR-P1 to tumour target cells and of the cytolysis of various target cells by NK

cells.

Figure 3a shows concentrations of free oligosaccharides giving 50% inhibition (IC_{50}) of binding of DLNN or IS with ^{125}I-sNKR-P1.

Figure 3b shows binding (cpm/cell) of ^{125}I-sNKR-P1 to suspensions of cells, against sNKR-P1 added (μg/ml). Symbols: ● , YAC-1 cells; ■ , B16S cells; ◆ , 1C21 cells; ▼, RNK-16 cells; ▽, glutaraldehyde-fixed RNK-16 cells; ◇, RNK-16 cells glutaraldehyde-fixed in the presence of IS disaccharide; O , P815 cells.

Figures 3c and d show IC_{50} values for inhibition of binding of ^{125}I-sNKR-P1 to various target cells.

Figures 3e and f show % specific lysis of various cells by fresh NK cells (e) or RNK-16 cells (f), at different effector:target cell (E:T) ratios. Symbols as for Figure 3b.

Figures 3g to j show inhibition of killing and k shows correlation of killing with inhibition of binding: symbols for inhibitors in panels g to k are: squares for sNKR-P1, circles for chondroitin sulphate A, diamonds for disaccharide IS, right side up triangles for GM2 tetrasaccharide, and upside down triangle for DLNN trisaccharide; closed, open and right-side shaded symbols are used for YAC-1, B16S and 1C21 cells, respectively; for results using fresh NK cells, small closed symbols are used to distinguish from results using RNK-16 cells.

Figure 4 demonstration of binding of sNKR-P1 to a

high-affinity carbohydrate ligand, GM2 glycolipid, in the presence (Figure 4a, closed circles) and absence (Figure 4a, open circles) of external calcium, and investigation of the involvement of NKR-P1 at the surface of fresh and activated NK cells in the calcium-independent conjugate formation with target cells (Figure 4b - conjugate forming NK cells as % of total).

Figures 4c to h show the results of flow immunocytometric analyses which were performed of the expression of NKR-P1, CD8 and CD5 antigens (solid lines) on fresh NK cells (panels c, e and g) and on activated NK cells (panels d, f and h). Dotted lines show fluorescence in control experiments using fluorescein-labelled rabbit anti-mouse immunoglobulin only.

Figure 5 shows levels of $InsP_3$ (panels a, d and g), $InsP_2$ (panels b, e and h), and free cytoplasmic calcium $[Ca^{2+}]_i$ (panels c, f and i) measured in RNK-16 effector cells after their interaction with YAC-1 target cells (panels a, b and c), or with liposomes containing oligosaccharide ligands for NKR-P1 (panels d, e and f), or with monoclonal anti-NKR-P1 antibody, or chondroitin sulphate A or the free disaccharide IS (panels g,h and i).

Figure 6 shows:

Figure 6a: binding (cpm/cell) of ^{125}I-sNKR-P1 to 5 x 10^5 YAC-1 cells or P815 cells, which had been treated with sialidase (S'ase) or heparinase I (H'ase)

or chondroitinase ABC (C'ase) or a mixture of sialidase and chondroitinase ABC (SC'ase) or a mixture of sialidase, chondroitinase ABC and heparinase I (SCH'ase). Control cells were treated with a mixture of the heat-inactivated enzymes.

Figure 6b: cytolyses (% specific lysis) of YAC-1 and P815 cells by fresh NK cells assayed after treatment of the tumour cells, as in panel a, with the glycosylhydrolases or the inactivated enzymes.

Figure 6c: cytolyses (% specific lysis) of YAC-1 or P815 cells by fresh NK cells assayed after exposure of the tumour cells to liposomes expressing lactose neoglycolipid (LAC), or GM2 glycolipid (GM2) or the neoglycolipid of IS disaccharide (IS).

Figure 6d shows the results of when plasma membranes of YAC-1 and P815 cells were subjected to SDS-polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose; strips were stained for protein (lanes 1) or overlaid with 125I-sNKR-P1 to reveal, by autoradiography (at 3 h), components bound (lanes 2); with YAC-1 cell membranes, additional strips (lanes 3, 4 and 5) were tested for binding after treatment with sialidase, chondroitinase ABC or heparinase I, respectively. The positions of Mr markers (kDa) are indicated.

Figure 6e shows: lanes 1, binding of 125I-sNKRP-1 was evaluated to lipids extracted from YAC-1 and P815 membranes resolved by TLC. Replicate strips, lanes 2,

were stained with primulin. O indicates position of application; GM1, GD1A, GD1B, GD1B lactone (GD1BL) and GT1B indicate the positions of authentic standards. GD1(A)L is a minor glycolipid component tentatively assigned as GD1A lactone. Binding to the relatively low abundance glycolipids GT1B and GD1B was of weak intensity (arrowed) in the 3 h autoradiogram.

Figure 7 shows radiobinding and inhibition of binding experiments showing the high affinity of sCD69 for sialylated and sulphated saccharides. The main panel shows binding of ^{125}I-sCD69 to lipid-linked oligosaccharides (neoglycolipids) immobilised on plastic microwells: protein bound (x10^{-4} cpm/well) v neoglycolipid added (pmol/well). The inset shows inhibition of the binding of sCD69 to IS neoglycolipid in the presence of selected saccharides.

Figure 8 shows results of radiobinding and cytotoxicity experiments indicating that CD69 is intimately linked to the cytolytic activity of human NK cells.

Figure 8A shows binding of ^{125}I-sCD69 to various leukaemia cell lines: protein bound (cpm/cell) v protein added (μg/ml).

Figure 8B shows natural killing (% specific lysis) of leukaemia cell lines at varoius effector:target (E:T) ratios.

Figure 8C shows the corrrelation between ^{125}I-sCD69 protein bound (cpm/cell) to luekaemia cells and

cytotoxicities at E:T ratio 128:1.

Figure 8D to G show concentration dependent activities of sCD69 (closed triangles) and SN disaccharide (open triangles) as inhibitors of natural killing of four NK sensitive cell lines: D - MOLT-4, E - K562, F - U937, G - Daudi.

Figure 9 shows surface expression of CD69 on human NK phenotype (CD56 class cells) is rapidly induced upon their incubation with NK-sensitive target cells or liposomes containing high affinity ligands for CD69 protein, and is inhibited in the presence of free oligosacccharide ligands and is delayed in cell populations that were first depleted in surface expression of CD69: A - % $CD56^+/CD69^+$ cells over time (h); B - % $CD3^+/CD69^+$ cells over time (h); C - % $CD56^+/CD69^+$ cells over time (h), the cells having been first depleted of $CD69^+$ cells by antibody-mediated complement lysis prior to incubation.

Figure 10 shows the rendering of NK-resistant-leukemic cell lines susceptible to natural killing by preincubating them with liposomes bearing oligosaccharide ligands for CD69. % specific lysis is shown against concentration of neoglycolipid added (nmol/100μl of liposomes): A - MOLT-4 cells; B - RAJI cells; C - IM-9 cells; D - KG-1 cells.

Figure 11 shows kinetics of uptake of neoglycolipid by cultured cell lines. Uptake of neoglycolipd into cells (% of cpm added) is shown

against time of incubation (h) for MOLT-4, RAJI, THP-1 and IM-9 cells.

Figure 12 shows apoptotic killing of NK susceptible target cells dependent on CD69-carbohydrate interactions.

(A) to (C): % specific lysis of (A) MOLT-4 cells, (B) u937 cells and (C) K562 cells is shown at ratios of E:T of 2, 8 and 32.

(D) to (F): Three types of assays were performed in Mg^{2+}/EGTA medium: in (D) the results of the 4 h ^{51}Cr release assays are shown at E:T ratios of 8:1 using PBMC (% specific lysis); in (E), results of the apoptosis assays by cytofluorimetry (% apoptotic cells) are shown; and in (F) results of the apoptotic DNA laddering assays are shown. Results are shown for the NK sensitive cell lines MOLT-4, U937 and K562 and the NK resistant cell lines RAJI designated R, THP-1 designated T, and IM-9 designated I. With MOLT-4 and U937 cells additional results are shown with reaction mixtures containing 10^{-5}M lactose (designated L) or the disaccharide SN (S) or 10^{-8}M sCD69 (C). Lanes designated (O) in panels (D) to (E) show results in the absence of inhibitors.

MODULATION OF ACTIVITY VIA NKR-P1

We have expressed in *Escherichia coli* a recombinant soluble form of NKR-P1 (referred to here as sNKR-P1) corresponding to the dimeric extracellular

portion of NKR-P1, and have shown that it is a calcium-dependent carbohydrate-binding protein with a range of monosaccharides bound that is different from those described for several other C-type lectins, and an unusually tight association with calcium[21].

Preliminary binding and inhibition studies with immobilized and free monosaccharides have shown (21) that the protein has a rather higher affinity of binding to β-N-acetyl-D-galactosamine than to β-N-acetyl-D-glucosamine, and a lower affinity toward α-L-fucose with IC_{50} values (concentrations giving 50 % inhibition of binding) of 0.6×10^{-7} M, 2×10^{-7} M and 2×10^{-6} M, respectively. We now identify some extremely potent saccharide ligands for NKR-P1, some with IC_{50} values in the range 10^{-9} to 10^{-12} M. These include oligosaccharide sequences of the blood group family, the ganglio family and glycosaminoglycans.

In addition, we provide evidence that interactions of such oligosaccharides on the target cell surface with NKR-P1 or closely related proteins at the NK cell surface are intimately involved in mechanisms of NK cell triggering that lead to cytolysis of target cells.

EXAMPLE 1

Expression of NKR-P1 and soluble fragments in COS cells

COS-7 cells were transfected with NKR-P1 cDNA in plasmid pCDm8 using DEAE-dextran procedure. Three days

after the addition of plasmid, cell surface proteins of nontransfected and transfected cells were iodinated by the glucose oxidase/lactoperoxidase protocol, lysed with Tris-buffered saline (TBS) containing 1% Triton X-100 and 1mM phenyl methyl sulfonyl fluoride (PMSF), and lysates were cleared by centrifugation at 100,000xg for 60 min.

Supernatants ($5x10^5$ cpm) were subjected to immunoprecipitation with monoclonal antibody, or affinity chromatography on 1 ml columns of monosaccharide-Sepharose. Columns were equilibrated in TBS containing 10 mM $CaCl_2$ and 0.1% Triton X-100 (TBS + C + T), and radioiodinated lysates applied in 1 ml of TBS + C + T. Columns were washed with the same buffer and 1 ml fractions were collected. Protein bound to GlcNAc-Sepharose was eluted with 0.5 M GlcNAc in TBS-C + T. Fractions were counted in a gamma counter, and aliquots (10^4 cpm) precipitated with 10% trichloracetic acid for analysis by SDS polyacrylamide gel electrophoresis under reducing and nonreducing conditions.

To prepare the soluble fragments NKR-071, NKR-081 and NKR-091, the NKR-P1 protein was isolated from Triton lysates of 10^9 transfected cells by affinity chromatography on GlcNAc-Sepharose as above, dialysed against TBS + C + T, and subjected, respectively, to the following treatments: (a) limited proteolysis (0.1 μg/ml trypsin or thrombin, 10 min at room temperature);

(b) limited proteolysis followed by deglycosylation with N-glycanase (according to the protocol suggested by enzyme manufacturer); or (c) subtilisin digestion (25 μg/ml subtilisin, 37°C, 1h). Protein products obtained by these treatments were subjected to affinity chromatography on GlcNAc-Sepharose and dialyzed against TBS + C.

EXAMPLE 2

Expression of Soluble NKR-P1 Fragments in Prokaryotic Cells

The entire protein coding sequence of NKR-P1 was subcloned from the original cDNA clone as a 0.8 kb HindIII-BgIII fragment (the HindIII site was filled and converted into an EcoRI site using an EcoRI linker) and transferred into pGEM-3Z vector, cut with EcoRI and BamHI, to obtain pNKR-124.

pNKR-141 contained the sequence coding for the entire extracellular portion of NKR-P1 starting with valine 65 transferred from pNKR-124 as a 145 bp HinfI-PflMI fragment. pNKR-141W had a single amino acid substitution in which tryptophan 115 was replaced by threonine during ligation at a PflMl site. pNKR-161 spanned the lectin-like domain of NKR-P1 from tryptophan 115, and was constructed by ligating an EcoRI linker (10 mer) to a unique PflMl site in pNKR-124 trimmed with T4 DNA polymerase. pNKR-171 and -191 are variants of the above constructs from which a

segment coding for the 8 most C-terminal amino acids has been removed.

This was achieved by opening the respective plasmids at a unique BsmI site, removing single-stranded extensions, and ligating an excission linker (#1144, New England BioLabs). Plasmids were reclosed, amplified *in vivo*, digested with BspMI and treated with mung bean nuclease to remove single-stranded extensions. The small EcoRI-BspMI fragment was ligated against filled ClaI site in the polylinker of EcoRI digested plasmid pGEM-7Zf(-) in order to recreate the stop codon. EcoRI/ HindIII inserts were transferred to vectors pINIIIompA2 or pMAL-2 (New England BioLabs) to obtain the expression plasmids pNKR-241, -261, -271 and -291, or pNKR-341, -341W or -391, respectively.

Proteins NKR-241 to -291 were produced in *Escherichia coli* strain JA221 by methods described previously, and purified by affinity chromatography on a GlcNAc-Sepharose column. Proteins NKR-341, -341W, or -391 were expressed in *Escherichia coli* strain NM522 using a protocol provided with the Protein Fusion and Purification System.

Briefly, 600 ml of LB medium with 100 μg/ml of amplicillin was inoculated with 6 ml of overnight culture containing the expression plasmids, and incubated in an orbital shaker at 300 rpm for 2 h at 37°C, and for an additional 30 min at room temperature. Cells were induced with isopropyl-β-D-thiogalactoside

(0.1 mM) and incubated for additional 3-4 h at room temperature. Cells were harvested by centrifugation in a Beckman J-6 centrifuge, resuspended in approximately 20 ml of extraction buffer (0.02 M Tris.HCl pH 8.0, 0.5 M NaCl, 10 mM EDTA, 10 mM 2-mercaptoethanol (2-ME), 1 mM NaN_3 and 1 mM PMSF), sonicated on ice for 4 x 30 s, and centrifuged at 15000xg for 10 min. Fusion proteins were isolated from the cleared bacterial extracts on an amylose-agarose affinity column according to a standard protocol (New England BioLabs).

Pools of each fusion protein (100 mg) were cleaved with Factor Xa at 23°C for 3 days, and NKR proteins separated from the maltose-binding protein by chromatography on DEAE-Sepharose column (2 x 15 cm), equilibrated in 10 mM Tris pH 8.0, 25 mM NaCl, 10 mM 2-ME, and eluted with a linear gradient of NaCl of 500 mM in 4 column volumes. Final purification was achieved by gel filtration on Sephacryl S-200 SF (0.5 x 120 cm) equilibrated in TBS + C containing 8 M urea and 10 mM 2-ME. NKR fragments were eluted in the second peak as determined by SDS polyacrylamide gel electrophoresis.

Proteins were renatured by a series of dialysis steps, first removing urea and then 2-ME and lowering the concentration of denaturants by half in multiple dialysis steps. Samples for antigenic analysis were collected after each dialysis. Finally, proteins were dialyzed against TBS + C containing 1 mM NaN_3 and stored in this buffer

EXAMPLE 3

High affinity saccharide ligands for NKR-P1

We have used as a reference compound the lipid-linked trisaccharide, DLNN (abbreviated designations are used in the text when referring to the saccharides shown in Table 1), with a terminal N-acetylglucosamine (Table 1), found to be bound by sNKR-P1. In particular, we have used DLNN to investigate the influence on NKR-P1 recognition of further elongation of the oligosaccharide backbone and peripheral substitutions with blood group-related monosaccharides, sialic acid and sulphate.

In initial chromatogram-binding experiments sNKR-P1 was found to bind to the LNT sequence where galactose is β1-3-linked to the N-acetylglucosamine of the DLNN sequence, but not to the LNNT sequence, where the galactose is β1-4-linked (Fig. 1a, lanes 1', 2' and 3'). However, in the presence of α1-3-linked fucose on the N-acetylglucosamine of LNNT, as in the Le^x antigen sequence LX5, there was binding (lane 2'). For the LNT backbone there was some enhancement of binding in the presence of α1-4-linked fucose as in the Le^a antigen sequence LA5 (lane 3').

The blood group H and Le^b sequences containing fucose α1-2-linked to galactose on the LNT and LA5 sequences as in H5 (lane 1') and LB6 (lane 3') were also bound. However, in the presence of the blood group A monosaccharide, α-N-acetyl-D- galactosamine on

the H and Le^b sequences as in A6 and A7, respectively, binding was not detected (results not shown). Thus, the blood group A monosaccharide 'masks' the binding sites on the Le^a and Le^b sequences.

In additional experiments (not shown) the glycolipid globoside with the oligosaccharide sequence GalNAcß1-3Galα1-4Galß1-4Glc was bound by NKR-P1, whereas Forssman glycolipid which consists of GalNAc joined by α1-3 linkage to the globoside sequence was not bound. Thus, the affinity of NKR-P1 for N-acetylgalactosamine[21] appears to be restricted to the ß-anomer (see also ganglio series saccharides below). An affinity for N-acetylneuraminic acid, α2-3-(but not α2-6-) linked, was also revealed for sNKR-P1, as there was binding to 3SL (lane 5') but not to 6SL (lane 4'), nor to the unsubstituted lactosyl backbone (lane 3').

Quantitative binding experiments showed that the binding of soluble NKR-P1 (sNKR-P1) to lipid-linked oligosaccharides immobilized on plastic microwells was saturable (panel b, inset). The binding data in Fig 1b and c, not only corroborated the chromatogram-binding data, but showed in addition that (i) α1-3-linked fucose confers a binding activity to the lactosyl backbone as in LX3, (ii) α2-3-linked N-acetylneuraminic acid potentiates the binding to LNT and to the Le^a and Le^x sequences as in 3SLT, 3SA5, and 3SX5, and (iii) α2-6-linked sialic acid on 6SLT is not bound; moreover binding is abrogated to the Le^x sequence by the

presence of α2-6-linked sialic acid as in 6SX5.

Sulphate on galactose (3-O-substituted) had an even greater potentiating effect on NKR-P1 binding to the Le^x and Le^a sequences than did the 3-linked sialic acid: compare 3SX3 with SUX3 and 3SA5 with SUA5 (Fig 1c). It was also apparent that oligosaccharide chain length has an influence on intensity of sNKR-P1 binding: the binding to SUX4 was greater than to SUX3, as was the binding to SUA5 greater than to SUA4. The presence of a 3-O-sulphated glucuronic acid joined by 3-linkage to the outer galactose of LNNT forming the HNK-1 antigen also conferred a considerable binding activity.

sNKR-P1 binding specificity is not confined to the blood group series of oligosaccharides but also encompasses the ganglio series (Fig 1c). As predicted from the monosaccharide-binding study (21), the glycolipid GA2 with a terminal β-N-acetyl-D-galactosamine was strongly bound. There was also binding, albeit less strongly, to this sequence in the presence of β1-3-linked galactose as in GA1. There was an enhancement of binding intensity when these two backbones were substituted with α2-3-linked sialic acid: compare GM1 with GA1, and GM2 with GA2. The presence of a second sialic acid, 2-8-linked, resulted in diminished binding: compare GD3 with GM3, GD2 with GM2, GD1B with GM1, and GT1B with GD1A. As with the blood group series, sulphate (3-O-substituted) on the

ganglio series backbones had a greater potentiating effect on sNKR-P1 binding than did the 3-linked sialic acid: compare SM3 with GM3, SM2 with GM2, SM1A with GM1, and SB1A with GD1A. Sulphatide (SULF) was also bound. The disulphated glycolipid SB2 with 3-O sulphate both on N-acetylgalactosamine and galactose was the most strongly bound among the ganglio series glycolipids.

Even stronger NKR-P1 binding was observed among oligosaccharides derived from heparin and chondroitin sulphate (Fig. 1d). The non-sulphated disaccharides OS and IVA *per se* supported binding, but the analogues containing variously sulphated hexosamines (2-N-sulphated glucosamine; 4-O-sulphated N-acetylgalactosamine; 6-O-sulphated N-acetylgalactosamine or N-acetylglucosamine) or 2-O-sulphated uronic acids (see Table 1) elicited pronounced binding. From the range of compounds tested, it appears that 4-O- and 6-O-sulphated N-acetylhexosamines are especially reactive. Among the disaccharides, the tri-sulphated IS from heparin was the most strongly bound. The strongest binding among all the oligosaccharides tested was to the hexasulphated heparin tetrasaccharide IS2. The binding activity of sNKR-P1 also encompasses phosphorylated oligosaccharides, as the 6-phosphorylated tetra- and pentasaccharides, M4P and M5P, supported binding.

Figure 1 shows binding of radioiodinated NKR-P1

protein to lipid-linked oligosaccharides. The oligosaccharide sequences and their abbreviations are in Table 1.

In a, lipid-linked oligosaccharides (neoglycolipids) (38,44), approximately 500 pmol of each, were chromatographed (38) on silica gel plates in solvent system chloroform/methanol/ water, 55/45/10 by volume (lanes 3 and 3′) or 65/35/8 by volume (other lanes); they were either stained chemically with orcinol (negative images shown in lanes 1 to 5), or overlaid with ^{125}I-labelled sNKR-P1 (a recombinant soluble, dimeric form of NKR-P1 expressed in E.coli; originally designated NKR-341), 5 x 10^6 cpm/ml (specific radioactivity 10^7 cpm/μg) (21) after treatment of the plates with 0.2 % (w/v) polymethacrylate, and binding was detected (38) by autoradiography (lanes 1′ to 5′). Lanes 1 and 1′ contained LNT and H5; lanes 2 and 2′ LNNT and LX5; lanes 3 and 3′, lactose (LAC), DLNN, LNT, LA5 and LB6; lanes 4 and 4′, 6SL; lanes 5 and 5′, 3SL; chromatography was upward.

In b, lipid-linked oligosaccharides (glycolipids or neoglycolipids) were immobilized on plastic microwells in the presence of the carrier lipids cholesterol and egg lecithin (40,41), and their interactions with ^{125}I-labelled sNKR-P1 protein were determined (21). In the inset, to demonstrate saturability of binding, ^{125}I-labelled sNKR-P1 was mixed

with unlabelled protein to give 10^6 cpm/μg, and applied at increasing levels to wells coated with the lipid-linked oligosaccharides GM2 and DLNN (2 pmol applied per well). In the main panel, the ascending parts of the binding curves are shown for the serial dilutions of selected lipid-linked oligosaccharides applied onto the wells, and overlaid with ^{125}I-sNKR-P1, 3 x 10^5 cpm per well. Symbols: □ , LNNT; ■ , LNT; ○ , DLNN; ▼ , GA2; ●, GM2; Δ , SUA5; ▲ , IS.

In c and d, the intensities of sNKR-P1 binding to lipid-linked oligosaccharides are compared by reading off, from the binding curves in panel b, and others not shown, the counts bound at coating levels of 64 pmol/well (panel c) and 8 pmol/well (panel d); results shown are means of duplicates with range indicated by error bars.

Oligosaccharides that were bound by sNKR-P1 in the solid-phase binding experiments inhibited the binding of this protein (Fig. 2a). When DLNN was used as the reference immobilized ligand, the N-acetylglucosamine-terminating and fucose-terminating oligosaccharides, DLNN and LA5, respectively, were approximately ten times more potent inhibitors of binding (IC_{50} 3 x 10^{-8} M and 2 x 10^{-7} M, respectively) than the monosaccharides, N-acetylglucosamine and fucose investigated earlier (21). Moreover, the sialylated oligosaccharide 3SA5 and the sulphated oligosaccharide SUA5 were approximately ten times and a

hundred times more potent inhibitors than LA5 with IC_{50} values 1.3 x 10^{-8} M (not shown), and 3 x 10^{-9} M, respectively. Also, mannose-6-phosphate (IC_{50} 6 x 10^{-8} M) was almost twenty thousand times more active than the unsubstituted mannose (IC_{50} 10^{-3} M (ref 21) and there was a further tenfold increase in potency (IC_{50} 5 x 10^{-9} M) in the presence of an extended mannosyl backbone as in M5P. The highest inhibitory activities were observed with the sulphated oligosaccharides from heparin and chondroitin sulphate (Fig 2a and b); the IC_{50} values for the most potent among these, IS and IS2, were 10^{-11} M and 1.3 x 10^{-12} M, respectively.

The IC_{50} values for a given oligosaccharide differed according to the immobilized oligosaccharide ligand (coat) used (Fig.3a): higher concentrations of the free oligosaccharides were required when the acidic compounds, GM2 glycolipid or IS neoglycolipid were used as coats rather than DLNN neoglycolipid. However, the hierarchies of inhibitory activities among the four oligosaccharides compared were the same.

When the inhibition studies were extended to polysaccharides (Fig.2c) it was observed that whole heparin glycosaminoglycan (despite its multivalence for NKR-P1 determinants) was only marginally more active per unit weight than the fully sulphated tetrasaccharide IS2 (IC_{50} 30 pg/ml and 40 pg/ml, respectively). This may be a reflection of the presence of undersulphated sequences (22) in the

glycosaminoglycan population. The keratan sulphate preparation had an activity comparable to that of heparin, indicating that 6-O-sulphated galactose (22,23) is also recognized by NKR-P1. The chondroitin sulphate preparations were superior inhibitors with IC_{50} values of 2, 5 and 10 pg/ml for chondroitin sulphates C, A and B, respectively. The high and low molecular weight forms of dextran sulphate, and hyaluronic acid were the least active of the polysaccharides tested, IC_{50} of 0.2 ng/ml, 1ng/ml and 1.8 ng/ml, respectively.

Figure 2 shows inhibition of the binding of radioiodinated sNKR-P1 to immobilized DLNN by free oligosaccharides and polysaccharides.

In a and c, DLNN neoglycolipid was coated onto microtiter wells (64 pmol applied per well), and the binding of ^{125}I-sNKR-P1 (5×10^4 cpm added per well) was measured in the presence of the indicated concentrations of saccharides. Regression curves were plotted from experimental points according to the formula $y = IC_{50} \div (x + IC_{50})$;
where $y = 1 - (\%\ \text{inhibition} \div 100)$, x = concentration of inhibitor, and IC_{50} is the concentration of inhibitor giving 50 % inhibition of binding.

Abbreviations for mono- or oligosaccharides are given in Table 1; those for the polysaccharides are: CA and CB for chondroitin sulphates A and B (bovine), respectively; CC for chondroitin sulphate C (shark); H for heparin (from porcine intestinal mucosa); DH and DL

are for high and low molecular weight dextran sulphates (average molecular mass 500 kDa and 5 kDa, respectively); HY, hyaluronic acid, all from Sigma Chemical Company; K, keratan sulphate (from bovine cornea) (23); the dotted curve in panel c is for the heparin tetrasaccharide IS2 expressed for comparison as μg/ml.

In b, the correlation is shown between the activities of the several oligosaccharides examined both in binding and in inhibition assays. Binding activities were calculated as ratios of counts bound to the specified immobilized oligosaccharides over those bound to immobilized DLNN (taken from Fig.1 at 64 or 8 pmol/well). Inhibitory activities of free oligosaccharides relative to that of DLNN were calculated as ratios of the IC_{50} for DLNN over those for the specified oligosaccharides; the IC_{50} values were derived from the inhibition curves shown in panel a, and others not shown.

Figure 3 shows comparisons of the potencies of oligosaccharides as inhibitors of the binding of radioiodinated sNKR-P1 to different oligosaccharide coats; and comparisons of the potencies of these oligosaccharides and those of unlabelled sNKR-P1 and chondroitin sulphate A (abbreviation CA) as inhibitors of the binding of ^{125}I-sNKR-P1 to tumour target cells and of the cytolysis of various target cells by NK cells.

In a, microwells were coated with DLNN neoglycolipid or GM2 glycolipid at 64 pmol/well, or IS neoglycolipid at 8 pmol/well (together with carrier lipids (40,41)) and the inhibition of binding of ^{125}I-sNKR-P1 determined[21] in the presence of various concentrations of the free oligosaccharides IS, GM2, IVA, and DLNN. The results are expressed as concentrations giving 50 % inhibition of binding (IC_{50}). In separate experiments (not shown) using a range of coating levels for the lipid-linked oligosaccharides (applying 4-32 pmol/well DLNN, 0.5-32 pmol/well GM2, or 0.5-8 pmol/well IS) it was observed that the IC_{50} values were negligibly affected.

In b, binding of ^{125}I-sNKR-P1 to suspensions of 5 x 10^5 cells was measured. Symbols: ● , YAC-1 cells; ■ , B16S cells; ◆ , 1C21 cells; ▼, RNK-16 cells; ▽, glutaraldehyde-fixed RNK-16 cells; ◇, RNK-16 cells glutaraldehyde-fixed in the presence of IS disaccharide; O , P815 cells. In separate experiments (not shown) sNKR-P1 binding to fresh NK cells was similar to that observed with RNK-16 cells.

In c and d, unlabelled sNKR-P1, chondroitin sulphate A (average molecular mass 15 kDa), and the oligosaccharides IS, GM2, IVA and DLNN were tested (over a range of concentrations) as inhibitors of the binding of ^{125}I-sNKR-P1 to the target cells YAC-1, B16S or 1C21; results were expressed as IC_{50}.

In e, cytotoxicities of the NK-sensitive YAC-1

and NK-resistant P815 cells by fresh NK cells, and in f, the cytotoxicities of YAC-1, P815 and also the NK-sensitive B16S and 1C21 cells by RNK-16 cells were assayed at different effector:target cell (E:T) ratios. Symbols for target cells are the same as in panel b.

In g, activities of the compounds shown in panel c were evaluated as inhibitors of killing of YAC-1 target cells by fresh NK cells, and in h to j, these same compounds were evaluated as inhibitors of the killing of YAC-1, B16S and 1C21 cells, respectively, by RNK-16 cells at E:T ratios given in the methods.

In panel k, the potencies of sNKR-P1, chondroitin sulphate A, and the oligosaccharides IS and GM2 are compared as inhibitors of sNKR-P1 binding to YAC-1, B16S and 1C21 cells (IC_{50} values taken from panel c) and of the killing of YAC-1 cells by fresh NK cells and killing of YAC-1, B16S and 1C21 cells by RNK-16 cells at E:T ratios given under Methods (IC_{50} values given are from experiments not shown).

Symbols for inhibitors in panels g to k are: squares for sNKR-P1, circles for chondroitin sulphate A, diamonds for disaccharide IS, right side up triangles for GM2 tetrasaccharide, and upside down triangle for DLNN trisaccharide; closed, open and right-side shaded symbols are used for YAC-1, B16S and 1C21 cells, respectively; for results using fresh NK cells, small closed symbols are used to distinguish

from results using RNK-16 cells.

METHODS

Fresh NK cells were isolated from spleens of male Fisher F344 rats by plastic adherence (45) following incubation in 200 U/ml of rIL-2 (Cetus Inc.) for 2 h. RNK-16, a NKR-P1$^+$ cell line derived from large granular lymphocytic leukaemia of rat[46] was grown in vitro in complete RPMI (medium containing 10 % fetal calf serum, L-glutamine and antibiotics) with added 25 μM 2-mercaptoethanol (RPMI/ME). The NK-sensitive target cell lines YAC-1 (T-cell lymphoma), B16S (melanoma), 1C21 (macrophage) and the NK resistant cell line P815 (mastocytoma) from the American Type Culture collection were cultured in complete RPMI.

For radiobinding studies, cells were washed three times in RPMI with 10 mM Hepes pH 7.4 (RPMI/ Hepes) and suspended at 5 x 10^6 cells/ml, and 100 μl cell suspensions (in triplicate in 1.5ml Eppendorf tubes) were mixed with ^{125}I-sNKR-P1 serially diluted in 10 μl of RPMI/Hepes, incubated at 23 °C for 1 h; cells were pelleted at 1000g for 5 mins, washed five times in 1 ml RPMI/Hepes, and bound radioactivity measured in a gamma counter.

In a parallel experiment RNK-16 cells were preincubated with IS disaccharide (10^{-7} M) in 10mM phosphate-buffered saline (PBS) or with PBS alone, fixed by adding glutaraldehyde to a final concentration

of 0.01% (v/v) and incubating for 10 min; cells were pelleted and washed five times with PBS, and any residual glutaraldehyde was quenched at 37°C for 30 min with complete RPMI; celss were washed three times with RPMI/Hepes, and used to measure ^{125}I-sNKR-P1 binding as described above.

For inhibition of cell binding experiments, 0.5 x 10^6 cells were mixed with ^{125}I-sNKR-P1 at concentrations giving half saturation of binding; serial dilutions of the inhibitors were included (total reaction volume 100 µl), and counts bound per cell determined as above. Spontaneous lysis of tumour cells by fresh NK cells and RNK-16 cell line was measured in triplicate over a range of E:T ratios, 0.5 to 64:1 by a standard ^{51}Cr release assay (47) at 37 °C for 4 h in total volume of 250 µl; spontaneous release of ^{51}Cr in the absence of effector cells was less than 5 %.

Cytotoxicity inhibition assays using YAC-1, B16S and 1C21 targets cells were performed at E:T ratios of 2:1, 4:1 and 16:1, respectively, in the presence of serial dilutions of inhibitors; total volume 250µl.

EXAMPLE 4

Saccharide-inhibitable sNKR-P1 binding to cells.

^{125}I-labelled sNKR-P1 bound to the tumour cell lines YAC-1, B16S and 1C21, which are known targets for NK cells[24]; there was negligible binding to the NK-

resistant P815 cell line (Fig. 3b). The binding to target cell lines was inhibitable with unlabelled sNKR-P1 (Fig 3c). Moreover, binding was most likely carbohydrate-mediated since it was inhibitable with saccharides, and the hierarchy of inhibitory activities of the four oligosaccharides was similar to that observed in inhibition experiments using immobilized oligosaccharides (cf Fig 3a and 3c).

Using YAC-1 cells, the IC_{50} values for the oligosaccharides were comparable with those using the glycosaminoglycan disaccharide IS as coat (Fig 3c); when B16S cells were used, IC_{50} values were reached with IS and GM2 oligosaccharides, but not with IVA and DLNN at the highest concentrations tested (10^{-4}M); when 1C21 cells were used, there was inhibition with IS but not with the other oligosaccharides. By an analogy with the difference between the IC_{50} values using immobilized GM2 and IS (Fig 3a), these results suggest that there may be some differences in the repertoire of acidic oligosaccharide ligands on each of the three target cell lines.

Some binding of sNKR-P1 was noted to the NK cell line, RNK-16 (Fig. 3b), as well as to fresh NK cells (results not shown), and additional binding sites were revealed when these cells were treated with the NKR-P1-inhibitory disaccharide IS, during fixation with glutaraldehyde, before the binding experiments [the gluteraldehyde fixation *per se* did not affect binding

as shown in Fig. 3b and there was no change in the level of sNKR-P1 binding to the P815 cells when these were similarly treated with IS and fixed with glutaraldehyde (not shown)]. If IS was washed away from the RNK-16 cells before fixation there was no enhancement of sNKR-P1 binding (not shown).

These observations suggest that there are reversible *cis*-interactions between membrane-associated saccharide ligands and endogenous NKR-P1 (or other carbohydrate-binding proteins) at the surface of killer cells, which mask some binding sites for exogenous NKR-P1. Both with fresh NK cells and RNK-16 cells the concentrations of oligosaccharide required for inhibition of sNKR-P1 binding were lower than those required with tumour cells, rather they were comparable to those with the DLNN coat (compare Fig. 3a and d).

EXAMPLE 5

Saccharide-inhibitable killing of target cells

sNKR-P1 protein did not elicit detectable lysis when added to YAC-1, B16S or 1C21 target cells at concentrations of 0.1 to 100 μg/ml (approximately 2 x 10^{-9} to 2 x 10^{-6}M) under conditions of NK lysis assay (not shown). However, lysis of YAC-1 cells by both fresh NK cells and RNK-16 cells, as well as the lysis of B16S and 1C21 cells tested by RNK-16 cells (Figs. 3e and f), was inhibitable by not only sNKR-P1 but also by saccharide ligands for this protein (Figs. 3g to j).

The inhibition data for the fresh NK cells and RNK-16 cells were very similar (shown for YAC-1 target cells in Figs. 3g and h). The IC_{50} values for the inhibition of killing as well as the relative activities of the individual inhibitors differed for the three target cells tested with RNK-16 cells (Figs. 3h, i and j), and the hierarchy of inhibitory activities was similar to that observed with inhibition of sNKR-P1 binding to these cells.

Thus as shown in Fig. 3k there was a striking correlation between the concentrations of oligosaccharides required for inhibition of sNKR-P1 binding to the target cells (Fig. 3c) and for inhibition of killing of a given target cell by both fresh NK cells and the RNK-16 cell line (Figs. 3g to j). Collectively these results clearly implicate NKR-P1 protein (and other proteins that may be present with binding specificities that are very similar to that of NKR-P1) in events leading to the tumor cell killing process.

In order to determine whether membrane-associated NKR-P1 on the killer cell is involved in conjugation with the target cells, we enumerated the conjugates formed in media lacking calcium and containing EGTA in order to prevent the lytic process. Although NKR-P1 is a calcium-dependent carbohydrate-binding protein, it associates unusually tightly with calcium, and we have observed only a 20% diminution in its binding to DLNN

neoglycolipid in the presence of EGTA (ref 21).

With higher affinity ligands, such as GM2, there was no detectable diminution of binding unless the protein was completely decalcified under nonphysiological conditions (Fig. 4a). Thus, under the conjugate assay conditions (in medium containing EGTA) it is deduced that NKR-P1-mediated interactions with carbohydrate ligands would not be greatly influenced.

When fresh NK cells were used, 11% formed conjugates with YAC-1 cells under these experimental conditions (Fig 4b); the number of conjugates formed was unchanged in the presence of sNKR-P1 or the disaccharide IS at 10^{-8} M and 10^{-7} M, respectively, which gave complete inhibition of ^{125}I-sNKR-P1 binding to the tumour cells, and complete inhibition of cytotoxicity (Fig. 3c and f). However, when activated NK cells were examined, the percentage of conjugate-forming cells was higher (26%); and when sNKR-P1 or the disaccharide IS were present in the reaction mixture, the proportion of conjugate-forming cells was reduced to that observed with the fresh NK cells (Fig 4b). As reported previously(18) the amount of NKR-P1 at the cell surface was considerably increased upon NK cell activation (Figs. 4c and d), whereas the amount of CD8 antigen was unchanged and CD5 antigen remained undetectable (Figs. 4e to h). Thus, two populations of conjugate-forming cells could be discerned among the activated NK cells: the first resemble those among

fresh NK cells where conjugate formation may involve other cell adhesion molecules (51), and the second, where NKR-P1 (or related proteins) mediates conjugate formation, and may account for the increased ability of these cells to form conjugates (52).

These experiments do not rule out the occurrence of readily dissociable conjugates mediated by NKR-P1 (analogous to the dissociable element of the DLNN-binding (21) discussed above) that would fail to be detected in the absence of calcium in the culture medium.

Figure 4 shows demonstration of binding of sNKR-P1 to a high-affinity carbohydrate ligand, GM2 glycolipid, in the absence of external calcium, and investigation of the involvement of NKR-P1 at the surface of fresh and activated NK cells in the calcium-independent conjugate formation with target cells.

In a, plastic microwells were coated with GM2 glycolipid (64 pmoles applied per well), and binding of ^{125}I-sNKR-P1 was measured, as described with reference to Fig. 1, after the following treatments of the radiolabelled protein: dialysis (4 days) against 10 mM Tris buffered sale (TBS) with 10 mM $CaCl_2$, pH 8 (TBS + C) (closed circles), or dialysis (4 days) against TBS containing 10 mM EGTA, pH 8 (TBS + E) (open circles), or for complete decalcification (21) dialysis against 0.1 M Tris-HCl pH 10 for 2 days followed by dialysis against TBS + E for 2 days (open squares); a duplicate

aliquot of the latter preparation was dialyzed back into TBS + C for 2 days (closed squares).

In b, the influence of sNKR-P1 and IS disaccharide on conjugation of fresh NK cells and activated NK cells with YAC-1 target cells was examined in control experiments in the absence of inhibitors, or in the presence of sNKR-P1 and IS tested as inhibitors. The results are presented as percent of NK cells forming conjugates (the mean ± S.D. from three determinations).

In c to h, flow immunocytometric analyses were performed of the expression of NKR-P1, CD8 and CD5 antigens (solid lines) on fresh NK cells (panels c, e and g) and on activated NK cells (panels d, f and h) to show the increased expression of NKR-P1 on the activated cells with a lack of expression of CD5 and unchanged expression of CD8 antigen. Dotted lines show fluorescence in control experiments using fluorescein-labelled rabbit anti-mouse immunoglobulin only.

METHODS

Fresh NK cells were prepared as described with reference to Fig. 3; to achieve maximum expression of cell surface NKR-P1 (ref 18), fresh NK cells were activated by culturing for 120 h in the presence of rlL-2, 1000 U/ml.

For measuring conjugate formation (50), YAC-1 cells were labelled with hydroethidine and effector

cells with calcein-AM (both from Molecular Probes); cells were washed, suspended in calcium-free PBS containing 10 mM $MgCl_2$, and 10^5 of each cell type were mixed in triplicate in the absence of additives or in the presence of sNKR-P1 (10^{-8}M) or IS disaccharide (10^{-7}M); the cells were pelleted at 4°C, heated at 37°C for 10 min, and the percentage of conjugate-forming cells determined by two-colour flow cytometry (FACSort, Becton Dickinson).

For cytofluorimetric analysis of surface expression of NKR-P1, CD8 and CD5 antigens, fresh or activated NK cells were incubated in PBS containing 3% bovine serum albumin and 0.1% NaN_3 and saturating concentrations of monoclonal antibodies 3.2.3. (anti-NKR-P1, ref. 18), or OX8, or OX19 (both from Serotec) at 23°C, washed and incubated at 23°C for 1 h in PBS with 0.1% NaN_3 containing fluorescein-labelled rabbit anti-mouse immunoglobulins (Cappel Inc., 1 μg/ml) washed with CA^{2+}/Mg^{2+} -free PBS containing 0.02% EDTA and analysed by flow cytometry.

EXAMPLE 6

Oligosaccharide-mediated activation of killer cells

In accord with earlier observations (19), $InsP_3$ and $InsP_2$ levels increased in RNK-16 cells upon exposure to YAC-1 target cells (Fig 5a, b) or when the membrane-associated NKR-P1 molecules were cross-linked with antibody (panels g and h). The responses to YAC-

1 cells were suppressed in the presence of sNKR-P1 and the heparin disaccharide IS (panels a and b) at concentrations which inhibited ^{125}I-sNKR-P1 binding to the target cells (10^{-8}M, and 10^{-7}M respectively). The elevation of free cytoplasmic calcium upon exposure to YAC-1 cells (panel 5c) was also suppressed in the presence of sNKR-P1, indicating that interactions of the membrane-associated NKR-P1 (or proteins that have similar binding specificities) with ligands at the tumour cell surface are intimately involved in the triggering of the cell activation process.

Liposomes containing the lipid-linked oligosaccharide ligands for NKR-P1, the disaccharide IS neoglycolipid and GM2 glycolipid, when added to the RNK-16 cells elicited pronounced increases in $InsP_3$ and $InsP_2$ to levels that were comparable with those observed after cross-linking of membrane-associated NKR-P1 with antibody (compare panels d and e with panels g and h). These responses elicited with the clustered oligosaccharides were inhibited if the corresponding free oligosaccharides were also included in the reaction mixtures (panels d and e).

Moreover, a dose-dependent elevation of free cytoplasmic calcium was observed in RNK-16 cells upon their exposure to GM2 liposomes (panel f). The effect depended on the density of the glycolipids incorporated into the liposomes; three levels of GM2 incorporation were investigated: at 0.2 nmol per 100μl of liposomes,

little change in free cytoplasmic calcium was detected; at 2 nmol (equivalent to the level in the phosphoinositide experiments in panels d and e), there was a more pronounced increase than at 20 nmol. This density-dependent effect on triggering of calcium rise by GM2 was also inhibited when GM2 oligosaccharide or sNKR-P1 were included in the reaction mixture (panel f).

In an experiment where RNK-16 cells were exposed to chondroitin sulphate A (10^{-7}M), the levels of $InsP_3$ and $InsP_2$ induced (panels g and h) were of the same order as those observed with the YAC-1 target cells shown in panels a and b (note differing scales in panels a, b and g, h); and there was a concommitant increase in cytoplasmic calcium (panel i).

Contrasting with the striking effects observed with the clustered oligosaccharide ligands presented on liposomes, when the RNK-16 cells were exposed to the free disaccharide IS, no changes were detected in the inositol phosphate levels (panels g and h) although there was a small (but reproducible) elevation in the overall level of cytoplasmic calcium (panel i).

Figure 5 shows involvement of carbohydrate-protein interactions in activation of NK cells. Levels of $InsP_3$ (panels a, d and g), $InsP_2$ (panels b, e and h), and free cytoplasmic calcium $[Ca^{2+}]_i$ (panels c, f and i) were measured in RNK-16 effector cells after their interaction with YAC-1 target cells (panels a, b and

c), or with liposomes containing oligosaccharide ligands for NKR-P1 (panels d, e and f), or with monoclonal anti-NKR-P1 antibody, or chondroitin sulphate A or the free disaccharide IS (panels g, h and i).

In a and b, the amounts of inositol phosphates generated were assayed (19) when myo-[^{3}H] inositol-labelled RNK-16 cells (5 x 10^6 in 0.25 ml of RPMI /ME) were mixed with an equal number of YAC-1 cells (0.25 ml) in the absence of inhibitors (closed circles), and in the presence of IS disaccharide (10^{-7} M) or sNKR-P1 protein (10^{-8} M) (open circles and open squares, respectively).

In d and e, inositol phosphates were assayed when NKR-16 cells (5 x 10^6 in 0.45 ml of RPMI/ME) were incubated with 50 μl of liposomes[48] consisting of 25 nmol each of cholesterol and egg lecithin with incorporated IS neoglycolipid (2.5 nmol, closed, right-side up triangles) or GM2 glycolipid (1 nmol, closed, upside-down triangles). In parallel experiments (corresponding open symbols) the incubation mixtures also contained the free oligosaccharides IS and GM2, (10^{-7} and 10^{-6} M, respectively).

In g and h, inositol phosphates were assayed[19] when myo-[^{3}H] inositol-labelled RNK-16 cells (5 x 10^6 in 0.5 ml RPMI/ME) were incubated with 0.5 μg of F(ab')$_2$ fragments[19] of the purified monoclonal antibody 3.2.3. and then cross-linked[18] by addition of 1 μg/ml F(ab')$_2$

of goat-anti mouse immunoglobulin (Capell Inc.) (closed squares), or chondroitin sulphate A, 10^{-7} M (closed diamonds), or IS disaccharide (10^{-7} M, closed triangles).

In c, f and i, baseline levels of free cytoplasmic calcium were assayed (49) for 5 min before the RNK-16 cells loaded with Indo-1 AM were exposed to any additives. The reactants in c consisted of 10^6 RNK-16 cells and the same number of YAC-1 cells in 1 ml of RPMI/ME in the absence of inhibitor (closed circles), or in the presence of 10^{-8}M sNKR-P1 which was added before the target cells (open circles).

In f, the reaction mixture consisted of 10^6 RNK-16 cells in 0.9 ml of RPMI/ME, and 100 μl of liposomes (50 nmol each of cholesterol and egg lecithin) and GM_2 glycolipid, 0.2 nmol (open right-side up triangles), or 2 nmol (closed right-side up triangles), or 20 nmol (closed upside down triangles), or 2 nmol (open upside down triangles) in the presence of GM2 tetrasaccharide (10^{-6}M) or sNKR-P1 protein (10^{-8}M); results with the GM2 tetrasaccharide are shown; those with sNKR-P1 were similar.

In i, the reaction mixture consisted of 10^6 cells in 1 ml of RPMI/ME and 10^{-7} M chondroitin sulphate A (closed diamonds) or IS disaccharide (closed triangles). At 15 min the incubation mixtures were exposed to 1 μM ionomycin to obtain maximum stimulation of calcium release from intracellular stores.

EXAMPLE 7

Mediation of NKR-P1 binding and cytolysis by sialyl and glycosaminoglycan-type ligands on target cells

The tumour cell lines were treated with sialidase, heparinase I, and chondroitinase ABC, singly or as mixtures. Results with the NK sensitive YAC-1, B16S and 1C21 cells were similar; those for YAC-1 and the NK-resistant P815 cells are shown in Fig. 6.

Both sNKR-P1 binding to and cytolysis of the NK-susceptible tumour cells were diminished by the sialidase and chondroitinase treatments; a mixture of the two enzymes had the greatest effect with a diminution of sNKR-P1 binding by approximately 60% and a diminution of specific cytolysis by approximately 50% of the control values, whereas the heparinase had little effect (panels a and b). In additional experiments (not shown) using heparinase III, no effects were observed on sNKR-P1 binding or cytolysis of the four tumour cell lines.

Preliminary investigations which include sNKR-P1 binding experiments using membrane preparations from the tumour cells subjected to polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose, and chromatogram binding experiments using glycolipid extracts from the tumour cells, indicate that the sialyl ligands on the NK-susceptible cells are predominantly gangliosides; on YAC-1 cells these have chromatographic properties and fragmentation

patterns in liquid secondary ion mass spectrometry consistent with those for GD1A, GM1, GM2, GT1A in order of decreasing abundance; the chondroitinase-susceptible ligands are polydisperse proteoglycans >200kDa - 60kDa (these will be described in detail elsewhere).

Roles for ganglioside and glycosaminoglycan-type ligands on target cells as mediators of natural killing could be clearly shown by performing the cytolysis assays (E:T ratios 16:1) after exposing the NK-resistant P815 cells to liposomes containing GM2 glycolipid or the heparin disaccharide IS or the lactose neoglycolipids (Fig. 6, panel c). Whereas in the absence of liposomes, or in the presence of liposomes containing lactose neoglycolipid, specific lysis of these cells was only 10%, this was increased to approximately 60% and 80% if the P815 cells were preincubated with the GM2 and IS liposomes, respectively. Thus, by pretreating with the NKR-P1 ligands on liposomes, these NK resistant cells were rendered almost as sensitive to cytolysis as the YAC-1 target cells at E:T ratio 16:1 (cf Fig. 3d). Preincubation of YAC-1 cells with GM2 and IS liposomes resulted in increased cytolysis of these cells also, as evidenced at E:T ratio of 4:1 (Fig. 6, panel c). If after the preincubation step any loosely associated liposomes were washed away from the tumour cells, the cytolysis of the tumour cells was increased, but to a lesser extent (panel c). If the preincubation step

with the liposomes was omitted, only a modest enhancement of the lysis of P815 cells was observed (to approximately 25% with the GM2 liposomes and 30% with the IS liposomes).

Figure 6 shows roles of membrane-associated sialyl and glycosaminoglycan type components on tumour cells as ligands for NKR-P1; the involvement of such components for susceptibility to cytolysis; and the induction of NK susceptibility to resistant tumour cells by incubating them with liposomes expressing the oligosaccharide ligands.

In a, binding of ^{125}I-sNKR-P1 was measured to 5 x 10^5 YAC-1 cells or P815 cells, which had been treated with sialidase (S'ase) or heparinase I (H'ase) or chondroitinase ABC (C'ase) or a mixture of sialidase and chondroitinase ABC (SC'ase) or a mixture of sialidase, chondroitinase ABC and heparinase I (SCH'ase), and the results were compared with binding to cells treated with a mixture of the heat-inactivated enzymes (Control).

In b, cytolyses of YAC-1 and P815 cells by fresh NK cells were assayed after treatment of the tumour cells, as in panel a, with the glycosylhydrolases or the inactivated enzymes.

In c, cytolyses of YAC-1 or P815 cells by fresh NK cells were assayed after exposure of the tumour cells to liposomes expressing lactose neoglycolipid (LAC), or GM2 glycolipid (GM2) or the neoglycolipid of

IS disaccharide (IS); the conditions for the liposome exposures were: preincubation with liposomes followed by washing of the tumour cells (P+W+); or preincubation with liposomes and no washing of the tumour cells (P+W-); or no preincubation and no washing of the tumour cells (P-W-); results are compared with cytolyses of the tumour cells in the absence of liposomes (control).

In d, plasma membranes of YAC-1 and P815 cells were subjected to SDS-polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose; strips were stained for protein (lanes 1) or overlaid with 125I-sNKR-P1 to reveal, by autoradiography (at 3 h), components bound (lanes 2); with YAC-1 cell membranes, additional strips (lanes 3, 4 and 5) were tested for binding after treatment with sialidase, chondroitinase ABC or heparinase I, respectively. The positions of Mr markers (kDa) are indicated.

In e, lanes 1, binding of 125I-sNKRP-1 was evaluated to lipids extracted from YAC-1 and P815 membranes resolved by TLC. Replicate strips, lanes 2, were stained with primulin. O indicates position of application; GM1, GD1A, GD1B, GD1B lactone (GD1BL) and GT1B indicate the positions of authentic standards. GD1(A)L is a minor glycolipid component tentatively assigned as GD1A lactone. Binding to the relatively low abundance glycolipids GT1B and GD1B was of weak intensity (arrowed) in the 3 h autoradiogram.

METHODS.

For glycosylhydrolase treatments the cultured tumour cells were washed three times with PBS; 10^7 cells were resuspended in 0.9 ml of 0.01 M phosphate buffer pH 6.5 containing 0.14 M NaCl; enzymes (0.1 U of *Arthrobacter sialidase*, or 2 U of chondroitinase ABC, both from Boehringer Mannheim, or 100 U of heparinase I or 100 U heparinase III from Sigma or mixtures of enzymes) were added to the cell suspensions in 0.1 ml of the same buffer, and incubated for 30 min at 37°C. As controls, the cells were incubated in the presence of the individual enzymes or mixtures of the enzymes which had been inactivated by incubating at 100°C for 5 min. After these treatments, the tumour cells were washed three times in RPMI, and used for binding assays with ^{125}I-sNKR-P1, or for cytotoxicity assays with fresh NK cells as described with reference to Fig. 3.

^{125}I-sNKR-P1 binding to cells or cytolysis of cells treated with heparinase III or with the four heat-inactivated enzymes was of the same order as with cells incubated in buffer only; the results shown as controls in panels a and b are means of the results obtained after treatment with the mixture of the inactivated sialidase, heparinase I and chondroitinase ABC.

In the liposome exposure experiments, YAC-1 or P815 cells (10^5 cells in 0.9 ml of complete RPMI) were mixed with 0.1 ml of liposome suspensions prepared as

described with reference to Figure 4, and containing 2 nmol of GM2 glycolipid or neoglycolipids of IS or lactose; the mixtures were incubated for 1 h at 37°C, and the liposome-treated tumour cells were used in the cytolysis assays after washing three times with complete RPMI, or without washing. Alternatively, the tumour cells were incubated for 1 h in the absence of liposomes and the liposomes were added to the cell suspensions immediately before the addition of effector cells. As controls, tumour cells were incubated in medium only.

Fresh NK cells were prepared and specific lysis of the tumour cells was assayed as described with reference to Fig.3 at E:T ratios 4:1 for YAC-1 cells and 16:1 for P815 cells. These ratios were selected by reference to the cytolysis curves in Fig. 3d in order to maximize visualization of enhanced killing both with the NK-sensitive YAC-1 cells and with the resistant P815 cells.

The results in a are means of duplicates with range indicated by error bars, and those in b and c, means of triplicates with standard deviations. In a and b, spontaneous release of ^{55}Cr by the tumour cells exposed to glycosylhydrolases and in c, exposed to liposomes, was less than 5% in the absence of effector cells.

For identifying components on tumour cells that are bound by 125I-sNKR-P1, aliquots of the membrane

preparations (85) were boiled in sample buffer (84), resolved in replicate lanes on 3-7% gradient SDS-polyacrylamide gels (30g of membrane protein per lane) and electrotransferred from gels onto nitrocellulose membranes, (0.45m, BioRad Laboratories, Hercules, CA), cut into parallel strips and one strip was stained for proteins (AuroDye, Amersham, UK). Other strips were blocked in 10mM Tris-HCl pH 8 containing 150mM NaCl, 10mM CaCl2, 5% BSA and 0.1% Tween 20, and incubated at 20°C for 1 h with the blocking reagent alone, or sialidase 10U/ml or heparinase I 100U/ml or chondroitinase ABC 1U/ml in 0.01M phosphate buffer pH 6.5, washed, and binding of 125I-sNKR-P1 determined under conditions described with reference to Fig. 2a.

After lipid extraction (88) of plasma membranes, extracts were desalted on Sep-Pak Vac C18 cartridges. (Millipore, Bedford, MA) and resolved by thin layer chromatography in chloroform/methanol/0.5% calcium chloride, 55/45/10 (v/v) using 4g extract per lane. Lipids were visualized by primulin staining (38), and binding of 125I-sNKR-P1 determined by autoradiography with reference to Fig 1a. Components bound were analysed directly on the chromatogram surface by liquid secondary ion mass spectrometry (44). The GD1B lactone was chemically synthesized (unpublished); a synthetic GD1A lactone was not available.

DISCUSSION OF NKR-P1 WORK

The results establish, first, that the membrane-associated NKR-P1 on NK cells is a key effector whose interactions with saccharide ligands on target cells result in killer cell activation as a lead to target cell killing, and second, that sialyl and glycosaminoglycan-type sequences constitute natural ligands on NK-susceptible tumour cells.

A role for NKR-P1 in the killing cascade could not be demonstrated by others when attempts were made (25) to block the killing using a monoclonal antibody 3.2.3 specific for NKR-P1.

From our results it is predicted that, depending on the target cell type, different classes of oligosaccharides: the blood group family, the ganglio family and glycosaminoglycans, on target cells may constitute ligands for NKR-P1.

Multispecificity for oligosaccharides, a feature shared with other endogenous lectins (6) (there are even overlaps in binding specificities) is unusually pronounced in the case of NKR-P1. The extensive cross-reactions observed with NKR-P1 may be a reflection of the high affinities of the CRD of this protein both for calcium and carbohydrates as shown in the present and earlier (21) studies. The results show that the highest affinities are towards the acidic oligosaccharides of chondroitin sulphate, heparin and keratan sulphate types, among the saccharides so far

investigated.

Some of the compounds identified here as NKR-P1 ligands, for example GM2 and heparin, have been shown previously to inhibit natural killing (27-29); and susceptibility of human leukaemia and lymphoma cells to NK lysis could be correlated with expression of GM2 (ref 30). In accord with the observations herein on the rat NK system using structurally defined oligosaccharides, others have reported (29) that in the murine NK system the inhibitory activities of heparin fractions correlate with their negative charge (i.e. degree of sulphation). No inhibitory effects with chondroitin sulphates A, B and C (glycosaminoglycan source and target cells not specified) were recorded by this group.

The results offer an explanation for the observations (31,32) that natural killing is inhibitable in the presence of mannose-6-phosphate (and hexose-6-sulphates), a phenomenon that was later shown (33,34) not to involve the mannose-6 phosphate receptor. In view of the considerable affinity of NKR-P1 for mannose-6-phosphorylated oligosaccharides, it will be interesting to investigate whether this protein has a role in the targeting of the mannose-6-phosphorylated (34) lysosomal enzymes within NK cell granules, and to deliver hydrolytic enzymes to the target cells in the course of the killing process.

Several of the NKR-P1 ligands identified in the

present study have been detected immunochemically at the surface of NK cells. These include asialoGM1 (GA1) in the mouse (35), and HNK-1 (36) and sialyl-Le^x (37) in the human. These antigens are not among the highest affinity ligands for NKR-P1 (Fig. 1c). Moreover, we have evidence that a proportion of the ligands on the NK cell surface are rendered cryptic through saccharide-inhibitable interactions with endogenous membrane-associated components. These features may serve to protect the NK cells from self-cytolysis.

The differing effects of exogenously applied monomeric (free) and clustered oligosaccharide ligands on NK activation are of special relevance to control mechanisms in the cytolytic cascade, and to design novel immunomodulatory drugs. The monomeric oligosaccharide ligands not only compete for NKR-P1 binding but they also inhibit the NK cell activation that is elicited by target cells and by clustered oligosaccharide ligands. In contrast, the clustered lipid-linked oligosaccharide ligands on liposomes can serve as 'decoys' that mimic the target cells and exhibit a density-dependent activation of NK cells: an increasing level of cytoplasmic Ca^{2+} is observed with increasing ligand density up to a critical density level, beyond which the Ca^{2+} levels are lower.

From the stand-point of drug design it is remarkable that striking negative or positive effects on the cytolytic process can be elicited simply with

the free or the lipid-linked forms, respectively, of a heparin disaccharide that lacks (22) anti-coagulant activity. The demonstration that NK-resistant tumour cells can be rendered susceptible by pre-treatment with clustered NKR-P1 ligands on liposomes offers powerful therapeutic possibilities for purging of selected cells.

MODULATION OF ACTIVITY VIA CD69

To initiate ligand identification studies, A soluble dimeric form of CD69 protein was prepared (referred to herein as sCD69) by expression of its extracellular portion in *Escherichia coli*. Initial studies indicate that the protein has an unusually tight association with calcium, a feature shared with NKR-P1 and that it is a carbohydrate-binding protein with N-acetyl-D-glucosamine and N-acetyl-D-galactosamine as strong monosaccharide inhibitors.

EXAMPLE 8

Production of soluble forms of CD69 in E. coli

Three proteins containing extracellular portions of CD69 antigen were prepared: CDA-401 encompasses the extracellular portion minus two amino acids starting with glycine 64; CDA-411 is further shortened and the cystein 68 is replaced by arginine; CDA-421 consists of the lectin-like domain.

The corresponding DNA fragments were transferred

into pMALc2 expression vector downstream from a unique EcoRI site; EcoRI linkers were used to correct the reading frames, and the expression plasmids were sequenced from double-stranded templates using pMAL primer and TaqTrack sequencing protocl (Promega, Madison, USA).

Soluble CD69 proteins were expressed as fusion proteins with the bacterial maltose-binding protein in *E. coli* strains NM522 and cleaved with Factor Xa. After cleavage and purification under denaturing conditions, soluble CD69 proteins were renatured while monitoring the appearance of CD69 epitopes recognised by two monoclonal antibodies. Ten cycles of Edman degradation were performed with the cleavage proteins to establish the presence of the correct amino acid sequence (an additional 4-5 amino acids from the expression vector were present).

The recombinant soluble CD69 used in the experiments described herein was CDA-401, and was purified by gel filtration on Sephacryl S-200 SF column, concentrated to 10 mg/ml, and stored at 4°C in Tris-buffered saline (TBS) containing 10 mM $CaCl_2$ and 1mM NaN_3.

EXAMPLE 9

Ligands for CD69

Exploratory binding experiments were performed with the radioiodinated soluble molecule, sCD69, and

several lipid-linked oligosaccharides (neoglycolipids), among them sequences containing the monosaccharides N-acetylglucosamine or N-acetylgalactosamine, which were shown to constitute recognition elements for this protein.

The N-acetylglucosamine-terminating trisaccharide DLNN (sequences of oligosaccharides are given in Table 2) was bound with a clear signal above background at a ligand coating level of 300 pmol per microwell (Fig. 7). Binding to this sequence was abrogated in the presence of a terminal β1-3 linked galactose as in LNT. The disaccharide lactose was also not bound (not shown); however, substantial binding was detected to the 6'-sialyl analogue, 6SL, but not to the 3'-sialyl analogue, 3SL, indicating recognition of α2-6 linked N-acetylneuraminic acid.

This conclusion was strikingly corroborated by the binding data using the disaccharide SN, which contains N-acetylneuraminic acid α2-6 linked to N-acetylgalactosamine. The sCD69 binding signal for SN at 1 pmol per well was equivalent to that with 300 pmol per well for DLNN which has an unsubstituted terminal N-acetylhexosamine. The additional recognition of 6-O substituted sulphate by CD69 was indicated by the binding data with the chondroitin sulphate disaccharide 6S which contains 6-O sulphated N-acetylgalactosamine, and the heparin disaccharide IS which contains 6-O sulphated glucosamine and two additional sulphates 2 O

substituted at uronic acid and N-substituted at glucosamine.

To gain insights into the relative affinities of oligosaccharides that differ in sialylation and sulphation patterns, a series of free oligosaccharides were investigated as inhibitors of sCD69 binding.

Initial experiments showed that inhibitory activity (for example, half maximal inhibitory concentrations, IC_{50}, values) is influenced by the strength of binding to the immobilized ligands: approximately tenfold higher concentrations of saccharide inhibitors are required when an acidic saccharide, such as the heparin disaccharide IS neoglycolipid, is used as the immobilized ligand compared with the non-acidic neoglycoprotein $GlcNAc_{23}$-bovine serum albumin which was the immobilized ligand used in our earlier study. Thus, when this neoglycoprotein or IS neoglycolipid (Table 2) are used as the immobilized ligands, the IC_{50} values for N-acetylgalactosamine are $8 \times 10^{-5}M$ and $2 \times 10^{-4}M$ and for N-acetylglucosamine $4 \times 10^{-5}M$ and $1 \times 10^{-4}M$, respectively.

In the present study the inhibition experiments have been done using the glycosaminoglycan-derived disaccharide IS as the immobilized ligand. Results with an extensive series of structurally defined oligosaccharides will be described in detail elsewhere. The results with selected compounds in Figure 7 inset

and Table 2 clearly show that some extremely high affinity ligands have been identified in the form of O glycosidic-type, N-glycosidic-type and glycosaminoglycan-type sequences.

The inhibition data with oligosaccharides Set 1 (Table 2) again show the masking effect of β1-3 linked galactose when added to the DLNN sequence as in LNT, resulting in a tenfold decrease in inhibitory activity from IC_{50} 2 x 10^{-5}M to 3 x 10^{-4}M. However, 6-O substitution of terminal N-acetylglucosamine with sulfate as in the keratan sulfate disaccharide K6 resulted in a greater than one hundred thousandfold increase in inhibitory activity (IC_{50} 8 x 10^{-11}M) compared with DLNN.

Results with oligosaccharides Set 2 highlight the preferential recognition of N-acetylneuraminic acid α2-6 linked to galactose over the α2-3 linked analogue. Thus, 6SL is more than ten thousand times more potent as an inhibitor of sCD69 binding than is 3SL, (IC_{50} 7 x 10^{-9} and 3 x 10^{-4}M, respectively).

Oligosaccharides Set 3 illustrate the potencies of glycoprotein oligosaccharides of N-glycosidic type as ligands for CD69. The biantennary oligosaccharide N2 with two terminal N-acetylglucosamine residues (IC_{50} 10^{-9}M) is almost ten thousand times more potent as an inhibitor than the monomeric DLNN. Here also the presence of a capping galactose on the outer chains as in G2 (IC_{50} 5 x 10^{-4}M) results in a dramatic loss of

inhibitory activity, by more than ten thousandfold, whereas the presence of outer chains substituted with α2-6 linked N-acetylneuraminic acid, as in SN (IC_{50} 2 x 10^{-11}M) increases the inhibitory acitivity almost ten millionfold over that of G2.

Oligosaccharides Set 4 illustrates CD69 recognition of oligosaccharides of O-glycosidic type. Here in contrast with the Sets 1 and 2 oligosaccharides, it is clear that substitution of the core N-acetylgalactosamine with β1-3 linked galactose as in disaccharide T enhances by about one hundredfold the inhibitory activity in relation to the non-substituted N-acetylgalactosamine, IC_{50} 3 x 10^{-6}M and 2 x 10^{-4}M, respectively. However, the potentiating effect of sialic acid α2-6 linked to the core monosaccharide is more manifest in the absence of the galactose residue, as the disaccharide SN (IC_{50} 6 x 10^{-12}M) is almost a hundredfold more active as an inhibitor than ST (IC_{50} 3 x 10^{-10}M). Thus an extraordinary enhancement of inhibitory activity by almost a hundred millionfold is manifest with SN over that with the unsubstituted monosaccharide.

Set 5, which consists of saccharides of chondroitin sulphate and heparin type shows that substitution of N-acetylhexosamines with uronic acids in the absence of sulphation, as in OS and IVA, results in an almost one thousandfold increase in inhibitory activity compared with the non-substituted

monosaccharides (IC_{50} 2 x 10^{-7}M, 5 x 10^{-7}M, respectively). It is also apparent that the 6-O sulphated form of the monosaccharide N-acetylgalactosamine NS (IC_{50} 4 x 10^{-8}M), although almost ten thousand times more active than the unsubstituted monosaccharide, is less potent by almost ten thousandfold than the 6'-sialyl analogue SN (cf. Set 4). The results presented in Table 2 and other results to be described elsewhere show that 2-O sulphation at uronic acid, 4-O sulphation at N-acetylgalactosamine or N-sulphation at glucosamine result in a relatively modest enhancement of inhibitory activity towards sCD69.

Bovine submaxillary mucin which is rich in the clustered O-glycosidic disaccharide NeuAcα2 6GalNAc (SN), is a potent inhibitor of sCD69 binding, IC_{50} 10 pg/ml. The commercially available glycosaminoglycans, heparin, chondroitin sulphates A, B and C which consist of linear oligosaccharide chains with average molecular masses in the range 1 2 kDa were less active per unit weight, IC_{50} 1, 7, 2 and 20 ng/ml, respectively, and a preparation of keratan sulphate peptidoglycan was even less active, 20% inhibition was recorded at the highest concentration tested, 100 ng/ml. From these results and other observations to be described elsewhere, we conclude that recognition elements that are clustered and terminally located, rather than internally located along linear oligosaccharide chains, are preferentially

bound by CD69.

Figure 7 shows radiobinding and inhibition of binding experiments showing the high affinity of sCD69 for sialylated and sulphated saccharides.

The main panel shows binding of ^{125}I-sCD69 to lipid-linked oligosaccharides (neoglycolipids) immobilized on plastic microwells. The neoglycolipids were serially diluted in methanol containing the carrier lipids cholesterol and egg lecithin 4(g of each per ml, applied onto micro wells and dried at 37°C. The wells were washed, blocked in the presence of 5% w/v bovine serum albumin and incubated for 2 h at 20oC with ^{125}I-sCD69. Thereafter the wells were washed and counted. The results shown are means of duplicate specific counts (less background counts) in wells lacking neoglycolipids.

The inset shows inhibition of the binding of sCD69 to IS neoglycolipid (60pmol applied per well) in the presence of selected saccharides. Results shown are means of duplicates with range indicated by error bars.

EXAMPLE 10

Natural killing involving saccharide-CD69 interactions

Because of the structural relationship between CD69 and several NK cell-associated proteins, and the clustering of the CD69 gene with those for several NK cell proteins (Testi *et al* 1994; Ziegler *et al* 1994a)

whether CD69 is involved in processes of natural killing in the human was investigated.

It was observed that ^{125}I-sCD69 binds to the cell lines MOLT4, K562, U937 and Daudi which are known to be susceptible to killing by human NK cells; there is little or no detectable binding to the NK-resistant cell lines, KG1, IM9, Raji and THP-1 (Fig. 8A). The hierarchy of binding intensities corresponded remarkably to the degrees of susceptibility of the cell lines to natural killing (Fig. 8B, C).

Furthermore, killing was inhibitable not only with the unlabelled sCD69 (IC_{50} 1.5 x 10^{-9}, 2.5 x 10^{-10}, 1.3 x 10^{-9} and 1 x 10^{-10} with MOLT-4, K562, U937 and Daudi cells, respectively) but also with the disaccharide ligand, SN (IC_{50} 1.5 x 10^{-6}, 1.2 x 10^{-7}, 1.2 x 10^{-8} and 1 x 10^{-6}M, respectively). The killing of K562 cells was completely inhibited, and with MOLT-4, U937 and Daudi cells, the maximum inhibition achieved was in the order of 80%.

The results strongly implicated CD69 involvement in the natural killing and indicated that interaction of CD69 with target cells is carbohydrate-mediated.

Figure 8 shows radiobinding and cytotoxicity experiments indicating that CD69 is intimately linked to the cytolytic activity of human NK cells.

(A) Binding of ^{125}I-sCD69 is shown to suspensions of cultured leukemia cell lines as described under Experimental Procedures. Results shown are means of

duplicates with range indicated by error bars.

(B) Natural killing of cultured cell lines. Cytotoxicities of the leukemia cell lines by PBMC was assayed by the ^{51}Cr release method over a range of effector:target (E:T) cell ratios as described in Experimental Procedures. Results are expressed as means of % specific cytotoxicity determined in triplicate with standard deviations.

(C) Correlation is shown between the binding of ^{125}I-sCD69 to the cultured leukemia cells (taken from panel A) and the cytotoxicities of these cells at E:T ratio 128:1 (taken from panel B).

(D) to (G) The concentration-dependent activities are shown of sCD69 (closed triangles) and SN disaccharide (open triangles) as inhibitors of natural killing of the four NK-sensitive cell lines as described under Experimental Procedures. Results shown are means of triplicates with standard deviations.

EXAMPLE 11

Target cell-dependent and carbohydrate-dependent induction of CD69 expression on CD56+ cells

CD69 is expressed on the surface of only a minor proportion of unstimulated NK cells (Lanier *et al* 1988), so CD69 expression on these cells was monitored (using CD56 as a marker antigen for NK cells) in the course of four hours. This is the period during which effector cells are incubated with target cells in the

standard short term natural killing assay.

At time zero less than 20% of the $CD56^+$ cells showed a detectable surface expression of CD69 (Fig. 9A). However, within thirty minutes of incubation with the NK-susceptible K562 cells, surface expression of CD69 was increased to 40%; and at one to two hours of incubation, over 50% of the NK phenotype $CD56^+$ cells were positive for surface CD69. Thereafter there was some decline in surface positivity to about 40% at 4 hrs, though a new wave of surface expression of CD69 followed.

The rapid induction of surface expression of CD69 was clearly target cell dependent as well as carbohydrate ligand-dependent as it was not observed when the NK-resistant cell line RAJI was used, and the induction was totally inhibited if the oligosaccharide ligand for CD69, SN, was included in the incubation mixture (Fig. 9A). Moreover, liposomes containing the lipid-linked oligosaccharide ligand, SN, mimicked the NK-susceptible target cells in eliciting surface expression of CD69, and with the same kinetics (Fig. 9A). Here also the surface induction of CD69 expression was abolished in the presence of the free oligosaccharide ligand, and no induction was observed with liposomes containing an irrelevant oligosaccharide, lactose. No induction of CD69 expression was detected amongst the $CD3^+$ cell population under the conditions of the natural killing

assay (Fig. 9B).

In separate experiments (Fig. 9C) where CD69+ cells were first depleted, there was a neglible increase in surface CD69 expression above control values during the four hours of incubation. However, by sixteen hours more than 80% of $CD56^+$ cells were positive for CD69. This delayed appearance of CD69 did not occur in the presence of free oligosaccharide SN. This suggests that the rapid surface induction of CD69 occurs among CD56 cells that already express at least minimal levels of surface CD69, but that after prolonged incubation there is appearance of newly synthesised CD69.

Figure 9 shows surface expression of CD69 on human NK phenotype (CD56 class cells) is rapidly induced upon their incubation with NK-sensitive target cells or liposomes containing high affinity ligands for CD69 protein, and is inhibited in the presence of free oligosacccharide ligands.

(A) Human PBMC were enriched for NK cells on Percoll gradients and incubated with the NK-sensitive K562 cells in the absence or presence of 10^{-5}M free SN disaccharide, or with SN liposomes in the absence or presence of 10^{-5}M the free disaccharide, or with the NK-resistant RAJI cells; as a negative control, the K562 cells were incubated in medium only. The cell surface expression of CD69 antigen on $CD56^+$ cells was monitored by two colour flow cytommetry as described under

Experimental Procedures. Results show % of the PBMC that were $CD56^+/CD69^+$.

(B) An experiment similar to that in (A) was performed to monitor the % of PBMC that were $CD3^+/CD69^+$. Here, K562 cells in the absence or presence of 10^{-5}M disaccharide SN, or RAJI cells were used. There was no induction of CD69 expression; only results with K562 cells are shown. Results are expressed as % of $CD3^+/CD69$ cells. Means of triplicates with standard deviations are shown.

(C) An experiment similar to that in panel (A) was performed but here the Percoll Isohypaque-enriched PBMC were first depleted of $CD69^+$ cells by antibody-mediated complement lysis prior to incubation with K562 cells in the absence or presence of 10^{-5}M of free disaccharide SN, or with Raji cells, and expression of CD69 on $CD56^+$ cells analyzed. Very little induction of surface CD69 expression was observed. Results with K562 cells in the absence and presence of SN oligosaccharide are shown: results with RAJI cells (not shown) were similar to those with K562 cells in the presence of inhibitor. Results are expressed as % $CD56^+/CD69^+$ cells (solid lines). Means in triplicate samples with standard deviations are shown.

EXAMPLE 12

Conferring susceptibility to natural killing by treatment with lipid-linked ligands of CD69

NK-resistant cell lines RAJI, IM9 and KG1 were exposed for one hour to liposomes containing a range of concentrations of the lipid-linked CD69 ligands SN or 6S; lactose neoglycolipid was used as a negative control (Fig. 10 B-D). Efficacy of uptake of the liposome-associated neoglycolipid(s) was found to be 40-70% in the different cell lines (Fig. 10D inset).

In cytotoxicity assays at E:T ratios of 16:1 the specific lysis levels reached, 40-65%, at ligand densities of 20nmol per 100μl of liposomes, compared favourably with those observed at (E:T 16:1) with the NK-sensitive cell lines U937, K562 and MOLT-4, 45-75% (cf Fig. 8B). In the presence of liposomes containing the control neoglycolipid, lactose, cytolysis of the NK-resistant cells was unaffected. The SN and 6S liposomes, but not the lactose liposome also resulted in a substantial enhancement of cytotoxicity of the NK-sensitive cell line MOLT-4; with these cells at E:T ratios of 1:1, specific cytotoxicity was increased from around 30% to greater than 70%. With all five cell lines investigated, the SN liposomes were more effective than the 6S liposomes in accordance with their potencies as CD69 ligands (see Table 2).

Figure 10 shows the rendering of NK-resistant-leukemic cell lines susceptible to natural killing by preincubating them with liposomes bearing oligosaccharide ligands for CD69.

(A) The NK susceptible cell line MOLT 4 and (B to

D) the NK resistant cell lines RAFJI, IM-9 or KG-1, were labeled with ^{51}Cr, and 10^4 cells in 90 μl of RPMI containing were mixed with 10 μl of liposomes (containing lipid-linked oligosaccharides at concentrations of 0.2 or 2 or 20 nmol per 100 μl of liposomes. After 1 h incubation at 37(C, PBMC were added in 150 (l RPMI at E : T ratios of 1:1 (MOLT 4 cells) or 16:1 (other cells), and cytotoxicity determined after 4 h as described under Experimental Procedures. Results are expressed as means of triplicates with standard deviations indicated by error bars.

Figure 11 shows kinetics of uptake of neoglycolipid by cultured cell lines.

Suspensions (5 x 10^4 cells in 90μl of RPMI) of the NK sensitive cell line MOLT 4 and of the NK resistant cell lines RAJI, THP 1 and IM 9 were incubated with 10 μl liposome preparations I (closed symbols) or II (open symbols) containing ^3H labeled lactose neoglycolipid (2 x 10^4 cpm) for the indicated times, and the uptake of the lipid-linked oligosaccharide by the cells (expressed as % of cpm added) was determined as described under Experimental Procedures. Results shown are means of duplicates of with ranges indicated.

In separate experiments (not shown) we observed that the NK-susceptibility of MOLT-4 cell line and of the NK-resistant cell lines is little affected when

CD69-bearing liposome preparations type II were used. In contrast, erythrocytes and normal lymphocytes took up approx. 40% and 25% respectively of the labelled lipid-linked oligosaccharides when liposome preparations II were used, but there was negligible uptake when liposome preparations type I were used. In natural killing assays, using ^{51}Cr-labelled red cells and ^{51}Cr-labelled lymphocytes, those treated with the type II liposomes (but not type I) showed specific lysis, up to approx. 40% and 50% respectively at E:T ratios of 16:1.

It is concluded that for different target cell types it may be necessary to explore liposome preparations with differing carrier lipid compositions in order to optimise the degree of transfer of the lipid-linked oligosaccharides. According to the above results, liposome preparations type II do not make normal red cells and lymphocytes vulnerable for natural killing, and so may be useful *in vivo* when it is desired to eliminate selectively abnormal cells, e.g. tumour, leukemia and virally infected cells.

EXAMPLE 13

Stimulation of apoptosis using ligands for CD69

To assess whether the engagement of CD69 on the killer cells with saccharides on target cells elicits apoptotic killing in the target cells, natural killing assays were performed in the absence of Ca^{2+} and the

presence of Mg^{2+}/EGTA thus inhibiting the non-apoptotic Ca^{2+} dependent natural killing pathway, under conditions that have been reported to be permissive to the apoptotic killing pathway in lymphocytes (Rouvier *et al* 1993).

At E:T ratios of 32:1, about half of the total specific cytotoxicity in MOLT-4 and U937 cells was Ca^{+2} independent (Fig. 12A, B). This cytotoxicity was negligibly affected by treatments of these two target cells with O glycoproteinase (not shown) or with NDV sialidase, but diminished by about a half by pretreatment with AU sialidase or N-glycanase. Ca^{2+} independent cytotoxicity was not observed with the NK-sensitive K562 cells.

To address the question of apoptosis further, additional cytotoxicity experiments in Mg^{2+}/EGTA were performed (Fig. 12D) as well as two types of apoptosis assay, cytofluorimetric and by electrophoresis of DNA, (panels E and F) in the presence of sCD69 or the disaccharide ligand for CD69 (SN), or an irrelevant oligosaccharide (lactose).

It was observed that the specific cytotoxicity in Mg^{2+}/EGTA elicited in PBMC was unaffected in the presence of lactose but was diminished by about a half both in the presence of oligosaccharide ligand SN or the soluble CD69 (panel D).

The results of the cytofluorimetric assays for apoptotic cells including the inhibition patterns with

oligosaccharide ligand, sCD69 and antibody fragment were in excellent agreement with the results of the cytotoxicity assays (panel E).

These and laddering analyses established the occurrence of apoptotic cell killing in the NK-sensitive cell lines, MOLT-4 and U937.

Figure 12 shows apoptotic killing of NK susceptible target cells dependent on CD69-carbohydrate interactions.

(A) to (C) Natural killing by PBMC was assayed by the standard Ca^{2+} containing medium (dotted lines) or in Ca^{2+}-free, Mg^{2+}EGTA medium that is permissive to apoptosis (solid lines) using the NK sensitive cell line MOLT-4, U937 and K562. With MOLT-4 and U937 cell lines, additional assays in the Mg^{2+}/EGTA medium were performed after treatment of the cells for 30 min at 37°C with NDV or AU sialidase, or with N-glycanase, or O-glycoproteinase. For comparability, the cells not subjected to enzyme treatments were incubated for 30 min at 37°C in the presence of buffer only. Results with O-glycoproteinase-treated cells (not shown) were indistinguishable from those with the untreated cells. Results are expressed as means of triplicates with standard errors.

(D) to (F) Three types of assays were performed in Mg^{2+}/EGTA medium: in (D) the results of the 4 h ^{51}Cr release assays are shown at E:T ratios of 8:1 using PBMC. In (E), results of the apoptosis assays by

cytofluorimetry, and in (F) those of the apoptotic DNA laddering assays are shown in which PBMC, which had been enriched for NK cells by the multistep procedure, and E:T ratios 1:1 were used, as described under Experimental Procedures. Results are shown for the NK sensitive cell lines MOLT-4, U937 and K562 and the NK resistant cell lines RAJI designated R, THP-1 designated T, and IM-9 designated I. With MOLT-4 and U937 cells additional results are shown with reaction mixtures containing 10^{-5}M lactose (designated L) or the disaccharide SN (S) or 10^{-8}M sCD69 (C). Lanes designated (O) in panels (D) to (E) show results in the absence of inhibitors.

In separate experiments (not shown), where the effector cells were excluded, the percentage of apoptotic cells recorded was less than 5% and DNA laddering was not observed and the results using a mixture of oligosaccharide SN with sCD69 were similar to when they were used separately. Results in (D) to (E) are expressed as means of triplicates with standard deviations indicated by error bars.

DISCUSSION OF CD69 WORK

It is herein established that CD69 on the surface of NK cells is intimately involved in the triggering of cytotoxicity by mechanisms that depend on engagement of this molecule with oligosaccharide ligands on target cells. In two NK susceptible target cells a

substantial proportion of the natural killing is apoptotic. A further new principle established by our experiments is the carbohydrate-dependent induction of CD69 expression on NK cells: a rapid induction with kinetics characteristic of translocation of the molecule to the cell surface from intracellular pools, followed by a delayed induction with kinetics characteristic of new protein synthesis.

A remarkable potentiation occurs in CD69 binding strength when the monosaccharides N-acetylgalactosamine or N-acetylglucosamine are substituted with α2-6 linked N-acetylneuraminic acid or 6-O sulfate; And there is further potentiation when the 6'-sialyl or 6'-sulfo monosaccharides are parts of oligosaccharides. Thus an IC_{50} value as low as 6×10^{-12}M, was observed for the disaccharide SN which occurs O-glycosidically linked to protein, 2×10^{-11}M for the chondroitin sulphate disaccharide 6S, and 8×10^{-11}M for the disaccharide K6 which occurs on keratan sulfate proteoglycan.

It is worthy of note that the 6-O sulfated N-acetylgalactosamine analogue of the K6 sequence (which we would predict to be among high affinity ligands for cd69) has been observed as a terminal sequence on the outer chains of n glycosidic oligosaccharides of pituitary hormones and other glycoproteins.

From the results presented in Table 2 (oligosaccharides sets 2 and 3) and those that will be described elsewhere, it is clear that CD69 recognizes

the 6'-sialyl motif *per se*, and not necessarily joined to N-acetylhexosamine. This motif occurs on the outer chains of N-glycosidically linked oligosaccharides joined to galactose; with the biantennary chain S2 an IC_{50} value of 2×10^{-11}M was recorded. The preferential recognition of 6'-sialyl over the 3'-sialyl motif in the case of CD69 is in sharp contrast to the observations with NKR-P1 of the rat which recognizes only the 3'-sialyl motif.

The mode of presentation of the recognition elements on macromolecular carriers has a profound influence on CD69 recognition. BSM on which the short O-glycosidic sequence SN occurs in the clustered state is a potent inhibitor of sCD69 binding, IC_{50} 0.01 ng/ml. In contrast, the glycosaminoglycans heparan sulphate and chondroitin sulphates a, b and c are poor inhibitors by two to three orders of magnitude although the disaccharide units that they contain are potent inhibitors when they are in the form of free oligosaccharides, and they elicit strong CD69 binding when presented in the clustered state. An extreme example is the peptidoglycan keratan sulphate which is rich in the linear repeating disaccharide sequence K6. The IC_{50} value for the free disaccharide is 8×10^{-11}M and yet this sequence is negligibly recognized by sCD69 when it is a part of the long oligosaccharides of this peptidoglycan.

EXPERIMENTAL PROCEDURES USED IN CD69 WORK

Saccharides, neoglycolipids and other glycoconjugates

Structures and designations of the saccharides investigated are given in Table 2.

The monosaccharides N-acetylglucosamine, N-acetylgalactosamine and their 6-O sulphated forms were from Sigma, as were the disaccharides lactose (LAC), 3'-sialyllactose (3SL), 6'-sialyllactose (6SL), and the chondroitin sulphate-derived disaccharides OS, 6S, and the heparin-derived disaccharides IVA, IIA and IS. Lacto-N-tetraose (LNT) was from Dextra. The trisaccharide DLNN was prepared by β-galactosidase treatment of lacto N neotetraose (Bezouska *et al* 1994a). The disaccharide K6 was isolated from bovine keratan sulphate by endo-β-galactosidase treatment (Scudder *et al* 1986), and the O-glycosidic disaccharides SN and T were isolated from bovine submaxilliary mucin (Chai, Feizi and Lawson unpublished observations) and an ovarian cyst glycoprotein, respectively, by non-reductive β-elimination (41). The O-glycosidic trisaccharide 6ST was a gift from Professor André Lubineau. The biantennary N-glycosidic type oligosaccharides N2, and G2 were gifts and S2 was purchased from Biocarb. Bovine submaxillary mucin, heparin from porcine intestinal mucosae, and chondroitin sulphates A and B (bovine) and chondroitin sulphate C (shark) were from Sigma. Keratan sulphate peptidoglycan was isolated from bovine cornea (Scudder

et al 1986).

Neoglycolipids (oligosaccharides conjugated to the aminophospholipid L1 2 dihexadecyl-*sn*-glycero-3-phosphoethanolamine) were prepared from lactose, DLNN, LNT, 3SL, 6SL, 6S and IS, and purified as described previously, using anhydrous conditions for derivatizing lactose, DLNN and LNT, and 5% v/v water for the other oligosaccharides.

^{3}H labeled neoglycolipids were prepared by substituting ^{3}H labeled sodium cyanoborohydride (specific activity 2.9×10^{13} Bq/mol) for the unlabeled equivalent. After the standard period of heating, caps of the reaction vials (except those of the neutral oligosaccharides) were removed to allow evaporation on the heating box over a period of several hours to improve the yields of neoglycolipids. The ^{3}H-labeled neoglycolipids of lactose and SN were isolated using phenylboronic acid columns and those of 6S and IS using C18 column.

In some experiments neoglycolipids were incorporated into liposomes made with (a) cholesterol and egg lecithin, 50nmol of each (Feizi *et al* 1978; Bezouska *et al* 1994b), referred to here as liposomes Type I or (b), phosphatidylserine and phosphatidylethanolamine, 50nmol of each (Correa-Freire *et al* 1984), liposomes type II, in 100 μl of RPMI. Both types of liposomes were prepared by sonication in a sonic water bath (Feizi 1978).

Glycosidases

Arthrobacter ureofaceans (AU) sialidase, Newcastle virus disease (NDV) sialidase and *Bacteroides fragilis* endo-β-galactosidase were from Boehringer Manneheim. Heparinase I and chondroitinase ABC were from Sigma. O-glycoproteinase (Abdullah *et al* 1992) was a gift.

Antibodies

Anti-α-fetoprotein (AFP-02) was provided by Dr. I. Hilgert, Institute of Molecular Genetics, Prague, and used as ascites at 1:1000 dilution.

Fluorescein-isothiocyanate (FITC)-labeled anti-CD69 (Leu-23), phycoerythrin (PE)-labeled anti-CD56 (Leu-19), and PE-anti-CD3 (Leu-4) were from Becton-Dickinson, and were used at dilutions recommended by the manufacturer.

Cells

Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Isopaque gradient centrifugation of heparinized blood; red cells were taken from the sediment. Where indicated, NK cell-enriched and lymphocyte-enriched fractions were obtained from PBMC by centrifugation on discontinuous Percoll gradients (Timonen *et al* 1981) or by a multistep procedure in which monocytes were removed by plastic adherence, B cells removed by passing through a

nylon wool column, and $CD3^+$ T cells removed using immunomagnetic beads (Borrego *et al* 1993). Human leukemia cell lines, MOLT-4, K562, U937, Daudi, RAJI, THP-1, IM-9 and KG-1, were obtained from European Tissue Culture Collection (UK) and cultured under standard conditions in complete RPMI with the exception of KG-1 cells, which were cultured in Iscove's DMEM with 20% (v/v) fetal calf serum.

For glycosidase treatments cultured cells, 10^6, were washed and resuspended in 50 μl of 10mM phosphate buffer pH 6.5 with 0.15 M NaCl, and mixed with 50 μl of this buffer containing 50 mU of AU sialidase, or 100 U of N-glycanase, or the amount of O-glycoproteinase able to cleave 80 μg of glycophorin A in 1 h (Abdullah *et al* 1992), or 50 mU of chondroitinase ABC. As controls, the heat-inactivated enzymes (heated at 100°C for 5 min) or buffer alone were used. After 30 min incubation at 37°C, cells were washed three times with complete RPMI.

For treatments with liposomes (Bezouska *et al* 1994b) cultured cells labeled with ^{51}Cr, 104 cells in 90 μl of complete RPMI, were mixed with 10 μl liposome preparations loaded with the indicated concentrations of neoglycolipids and incubated for 1 h at 37°C.

For determining the kinetics of uptake of neoglycolipids by cells, 5 x 10^4 cells in 90 μl of complete RPMI were mixed with 10 μl of liposome preparations containing ^3H-labeled neoglycolipids, 0.2

nmol (2×10^4 cpm) and incubated for the indicated times at 37°C, and chilled on ice. Cells were washed with ice cold serum-free RPMI and the cell pellets obtained after centrifugation at 500g, were counted by liquid scintillation. Radioactive counts in control tubes which contained labeled liposomes in the absence of cells only was less than 0.1% of added counts.

Radiobinding assays

sCD69 binding to neoglycolipids: Neoglycolipids were immobilized on 96 well flat bottom plastic microwells (Immulon 1, Dynatech Inc) in the presence of the carrier lipids cholesterol and egg lecithin (40,41), and binding of ^{125}I-sCD69 (10×10^6 cpm/μg, 10^5 cpm/well) was determined as described previously (Bezouska *et al.* 1994a). For inhibition assays, the microwells were coated by applying 32 pmol/well of IS neoglycolipid, and the binding of ^{125}I-sCD69 (10^5 cpm/well) was measured in the presence of indicated concentrations of oligosaccharides.

sSC69 binding to cells: Cultured cells were washed in RPMI with 10 mM Hepes pH 7.4, suspended at 5×10^6 cells /ml, and ^{125}I-sCD69 binding to 100 μl aliquots in 1.5ml Eppendorf tubes was determined as described previously (Bezouska 1994b). Nonspecific binding, estimated in the presence of a thousand fold excess of unlabelled protein (always amounting less than 1 % of the experimental counts) was subtracted.

Cytotoxicity Assays

Standard four hour cytotoxicity assays (Brunner *et al.* 1968) were performed in 96 well V-bottom plates (Falcon) in complete RPMI medium using PBMC as effectors and ^{51}Cr-labeled cultured leukemia cells as targets, total reaction volume 250 μl.

Four hour cytotoxicity assays in the absence of extracellular calcium (Kojima *et al* 1994) were performed in Ca^{2+}-free medium prepared by mixing Ca^{2+}-deficient Hank's balanced salt solution containing 1mM Mg^{2+} with dialyzed fetal calf serum (final concentration 10 % v/v) containing 0.1mM EGTA (Mg^{2+}/EGTA medium).

Analysis of changes in CD69 expression

PBMC enriched for NK cells on Percoll gradients, 10^5 cells in 0.5ml complete RPMI, were mixed with 10^5 target cells or 100 μl of liposomes suspended in 0.5ml medium, with or without the indicated final concentrations of oligosaccharides, and incubated at 37°C.

At the end of the indicated incubation periods, cells were spun and kept on ice in 10 mM phosphate buffered saline pH 7.4 (PBS) with 5 % (w/v) BSA and 0.1% NaN_3 (staining buffer). Cells were incubated with 1 mg/ml of an irrelevant monoclonal antibody (anti-α-fetoprotein) in staining buffer for 30 min, followed by a further 30 min incubation with FITC-anti-CD69 and PE anti-CD56 antibodies, or with FITC-anti CD69 and PE-

anti-CD3 antibodies; thereafter the cells were washed three times in staining buffer and the expression of cell surface antigens was determined by flow cytometry (FACScan, Becton-Dickinson) equipped with three-colour fluorescence. Ten thousand events were acquired, and dead cells were gated out by means of propidium iodide staining.

The results were expressed as % cells stained with CD56 or with CD3, or as relative cell immunofluorescence with anti CD69, or as % double-positive cells.

In certain experiments the effector cell preparations were depleted of cells with surface expression of CD69 by incubation with saturating concentrations of BL-FB/B1 monoclonal antibody plus complement for 1 h (Correa-Freire *et al* 1984); dead cells were removed by Ficoll-Isopaque gradient centrifugation and the remaining cells washed three times with a complete RPMI.

Apoptosis assays by flow cytometry

Unlabelled target cells in the presence or absence of various inhibitors were mixed, under conditions identical to those in the calcium-independent cytotoxicity assay, with PBMC enriched for NK cells by the multistep procedure; total reaction volume was 250 μl. At the end of the 4 h incubation period, cells were spun, and DNA fragmentation was

visualized (Sgonc *et al* 1994) by terminal deoxynucleotidyl transferase-(TdT) mediated dUTP-FITC nick end labeling of DNA fragmentation sites in nuclei, and FACS analysis.

Five thousand events were acquired per experiment; all cells with a relative fluorescence of FITC-dUTP greater than 10^2 were scored as apoptotic, and results expressed as % apoptotic cells.

Analysis of DNA fragmentation

For detection of apoptotic DNA fragmentation (laddering pattern), $5x10^5$ target cells in 0.4 ml of mg^{2+}/EDTA medium were mixed with the equal number of PMBC, enriched for NK cells by the multistep procedure (0.4ml), and 200 μl of medium containing various inhibitors or medium alone, and incubated at 37°C for 4h. At the end of the incubation period, cells were spun and resuspended in lysis buffer containing 10 mM Tris-HCl pH 7 with 10 mM EDTA, 0.2 % Triton X-100, and 0.1 ug/ml of proteinase K. DNA preparations were obtained (Eischen *et al* 1994) and analyzed by electrophoresis in 1.2 % agarose gel (Sambrook *et al* 1989).

TABLE 1

Monosaccharides and oligosaccharides investigated for interactions with NKR-P1

The saccharides shown were investigated as

neoglycolipids i.e. oligosaccharides conjugated (38) to the aminophospholipid L-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine, except those under the headings *Gangliosides* and *Sulphated Glycolipids* which were natural glycolipids, and the compounds 3SX5 and 6SX5 under *Sialylated Type 2* which were chemically synthesized glycosphingolipids. A proportion of these saccharides (indicated in Fig. 2a and b) and mannose-6-phosphate were also investigated as free saccharides. Descriptions of the saccharides under the headings *Neutral*, *Sialylated*, and *Sulphated* (*Types 1 and 2*, of the blood group family) are given in refs 21, 39-41, except 3SL and 6SL, which were from Sigma; those for *Gangliosides, Sulphated Glycolipids and Phosphorylated* saccharides are in ref 42, except the free oligosaccharides of GM2 and GM1 (given in ref 43); the tetrasaccharide IS2 was isolated from porcine intestinal heparin (Sigma) after digestion with heparinase I (EC 4.2.2.7., Sigma) followed by strong ion exchange HPLC (unpublished); the chondroitin sulphate and heparin disaccharides were from Dextra Laboratories.

Abbreviations for monosaccharides: Fuc, fucose; Gal, galactose; GalNAc, N-acetyl- galactosamine; Glc, glucose; GlcN, glucosamine; GlcNAc, N-acetylglucosamine; GlcA, glucuronic acid; IdoA, Iduronic acid; Man, mannose; NeuAc, N-acetylneuraminic acid; UA, uronic acid (glucuronic or iduronic

unspecified); ΔUA, 4,5-unsaturated uronic acid.

TABLE 2

Monosaccharides and oligosaccharides investigated for activities as inhibitors of binding of CD69 (to IS neoglycolipid)

Details for several of the oligosaccharides are given in Table 1 (DLNN, LAC, 3SL, 6SL, the chondroitin sulphate disaccharides, Chon O-S, 6-S (the latter two are abbreviated here as OS and 6S), the heparin disaccharides HEP IV-A, II-A and I-S (abbreviated here as IVA, IIA and IS, respectively), the monosaccharides N-acetylglucosamine (C), N-acetylgalactosamine (N) and their 6-O sulphated forms (SC and NS) were from Sigma, as were Chon 2,6S and 2,4,6S. Lacto-N-tetraose (LNT) was from Dextra. The trisaccharide DLNN was prepared by β-galactosidase treatment of lacto N neotetraose (Bezouska *et al* 1994a). The disaccharide K6 was isolated from bovine keratan sulphate by endo-β-galactosidase treatment (Scudder *et al* 1986), and the O-glycosidic disaccharides SN and T were isolated from bovine submaxilliary mucin (Chai, Feizi and Lawson unpublished observations) and an ovarian cyst glycoprotein, respectively, by non-reductive β-elimination (41). The O-glycosidic trisaccharide 6ST was a gift from Professor André Lubineau. The biantennary N-glycosidic type oligosaccharides N2, and G2 were gifts and S2 was purchased from Biocarb.

Bovine submaxillary mucin, heparin from porcine intestinal mucosae, and chondroitin sulphates A and B (bovine) and chondroitin sulphate C (shark) were from Sigma. Keratan sulphate peptidoglycan was isolated from bovine cornea (Scudder *et al* 1986).

TABLE 1. Monosaccharide and oligosaccharide sequences investigated in the present studies in the free or lipid-linked form

Designation	Abbvn	Structure
Neutral type 1		
Lacto-*N*-tetra	LNT	Galβ1-3GlcNAcβ1-3Galβ1-4Glc
H penta	H5	Galβ1-3GlcNAcβ1-3Galβ1-4Glc |1,2 Fucα
Le^a penta	LA5	Galβ1-3GlcNAcβ1-3Galβ1-4Glc |1,4 Fucα
Le^b hexa	LB6	Galβ1-3GlcNAcβ1-3Galβ1-4Glc |1,2 |1,4 Fucα Fucα
A hexa	A6	GalNAcα1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc |1,2 Fucα
A hepta	A7	GalNAcα1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc |1,2 |1,4 Fucα Fucα
Sialylated type 1		
3'-sialyllacto-*N*-tetra	3SLT	NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc
6'-sialyllacto-*N*-tetra	6SLT	Galβ1-3GlcNAcβ1-3Galβ1-4Glc |2,6 NeuAcα
3'sialyl Le^a penta	3SA5	NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc |1,4 Fucα
Sulphated type 1		
3'-sulphated Le^a tri	SUA3	Galβ1-3GlcNAc |3 |1,4 HSO_3 Fucα
3'-sulphated Le^a tetra (penta)	SUA4 (SUA5)	Galβ1-3GlcNAcβ1-3Gal(β1-4Glc) |3 |1,4 HSO_3 Fucα
Neutral type 2		
Lactose	LAC	Galβ1-4Glc
Degalactosylated lacto-*N*-neo-tetra	DLNN	GlcNAcβ1-3Galβ1-4Glc
Lacto-*N*-neo-tetra	LNNT	Galβ1-4GlcNAcβ1-3Galβ1-4Glc
Le^x tri (glucosyl)	LX3	Galβ1-4Glc |1,3 Fucα
Le^x penta	LX5	Galβ1-4GlcNAcβ1-3Galβ1-4Glc |1,3 Fucα
Sialylated type 2		
3'-sialyllactose	3SL	NeuAcα2-3Galβ1-4Glc
6'-sialyllactose	6SL	NeuAcα2-6Galβ1-4Glc
Sialyl Le^x tri (glucosyl)	3SX3	NeuAcα2-3Galβ1-4Glc |1,3 Fucα
3'-sialyl Le^x penta	3SX5	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc |1,3 Fucα
6'-sialyl Le^x penta	6SX5	NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc |1,3 Fucα

TABLE 1 cont.

Sulphated type 2		
HNK-1	HNK1	GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc |3 HSO_3
3'-sulphated Le^x tri	SUX3	Galβ1-4GlcNAc |3 |1,3 HSO_3 Fucα
3'-sulphated Le^x tetra	SUX4	Galβ1-4GlcNAcβ1-3Gal |3 |1,3 HSO_3 Fucα
Gangliosides		
GM_3	GM3	NeuAcα2-3Galβ1-4Glc
AsialoGM_2	GA2	GalNAcβ1-4Galβ1-4Glc
GM_2	GM2	GalNAcβ1-4Galβ1-4Glc |2,3 NeuAcα
AsialoGM_1	GA1	Galβ1-3GalNAcβ1-4Galβ1-4Glc
GM_1	GM1	Galβ1-3GalNAcβ1-4Galβ1-4Glc |2,3 NeuAcα
GD_3	GD3	NeuAcα2-8NeuAcα2-3Galβ1-4Glc
GD_2	GD2	GalNAcβ1-4Galβ1-4Glc |2,3 NeuAcα2-8NeuAcα
GD_{1a}	GD1A	NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glc |2,3 NeuAcα
GD_{1b}	GD1B	Galβ1-3GalNAcβ1-4Galβ1-4Glc |2,3 NeuAcα2-8NeuAcα
GT_{1a}	GT1A	NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glc |2,8 |2,3 NeuAcα NeuAcα
GT_{1b}	GT1B	NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glc |2,3 NeuAcα2-8NeuAcα
Sulphated glycolipids		
Sulphatide	SULF	Gal |3 HSO_3
SM3	SM3	Galβ1-4Glc |3 HSO_3
SM2	SM2	GalNAcβ1-4Galβ1-4Glc |3 HSO_3
SM1a	SM1A	Galβ1-3GalNAβ1-4Galβ1-4Glc |3 HSO_3
SB2	SB2	GalNAcβ1-4Galβ1-4Glc |3 |3 HSO_3 HSO_3
SB1a	SB1A	Galβ1-3GalNAcβ1-4Galβ1-4Glc |3 |3 HSO_3 HSO_3

TABLE 1 cont.

Chondroitin sulphate		
Chon O-S	OS	ΔUA1-3GalNAc
Chon 4-S	4S	ΔUA1-3GalNAc |4 HSO_3
Chon 6-S	6S	ΔUA1-3GalNAc |6 HSO_3
Heparin		
Hep IV-A	IVA	ΔUA1-4GlcNAc
Hep III-A	IIIA	ΔUA1-4GlcNAc |2 HSO_3
Hep II-A	IIA	ΔUA1-4GlcNAc |6 HSO_3
Hep III-S	IIIS	HSO_3 |2 ΔUA1-4GlcN |2 HSO_3
Hep I-S	IS	HSO_3 |2 ΔUA1-4GlcN |2 |6 HSO_3 HSO_3 HSO_3
Hep tetra	IS2	HSO_3 |2 |2 ΔUA1-4GlcNα1-4IdoAα1-4GlcN |2 |6 |2 |6 HSO_3 HSO_3 HSO_3 HSO_3
Phosphorylated		
Mannose-6-phosphate	MP	Man |6 H_2PO_3
Tetramannose phosphate	M4P	Manα1-3Manα1-3Manα1-2Man |6 H_2PO_3
Pentamannose phosphate	M5P	Manα1-3Manα1-3Manα1-3Manα1-2Man |6 H_2PO_3

TABLE 2. Potencies of saccharides as inhibitors of the binding of sCD69 to IS neoglycolipid

Designation		Sequence	IC_{50} (M)
	C	GlcNAc	2×10^{-4}
	DLN	GlcNAcβ1-3Galβ1-4Glc	2×10^{-5}
Set 1	LNT	Galβ1-3GlcNAcβ1-3Galβ1-4Glc	3×10^{-4}
	SC	GlcNAc |6 HSO_3 GlcNAcβ1-3Gal	3×10^{-8}
	K6	|6 HSO_3	8×10^{-11}
	LAC	Galβ1-4Glc	$>10^{-3}$
	3SL	Galβ1-4Glc |2,3 NeuAcα	3×10^{-4}
Set 2	6SL	Galβ1-4Glc |2,6 NeuAcα	7×10^{-9}
	6SLN	Galβ1-4GlcNAcβ1-3Galβ1-4Glc	2×10^{-9}
	N2	GlcNAcβ1-2Manβ1-6\ Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-2Manβ1-3/	10^{-9}
	G2	Galβ1-4GlcNAcβ1-2Manβ1-6\ Manβ1-4GlcNAcβ1-4GlcNAc Galβ1-4GlcNAcβ1-2Manβ1-3/	5×10^{-4}
Set 3	S2	NeuAcα |2,6 Galβ1-4GlcNAcβ1-2Manβ1-6\ Manβ1-4GlcNAcβ1-4GlcNAc Galβ1-4GlcNAcβ1-2Manβ1-3/ |2,6 NeuAcα	2×10^{-11}
	N	GalNAc	2×10^{-4}
	T	Galβ1-3GalNAc	3×10^{-6}
Set 4	SN	GalNAc |2,6 NeuAcα Galβ1-3GalNAc	6×10^{-12}
	6ST	|2,6 NeuAcα	3×10^{-10}
	NS	GalNAc |6 HSO_3	4×10^{-8}
	OS	ΔUA1-3GalNAc	2×10^{-7}
	6S	ΔUA1-3GalNAc |6 HSO_3	2×10^{-11}
Set 5	2,6S	ΔUA1-3GalNAc |2 |6 HSO_3 HSO_3	1×10^{-11}

TABLE 2 cont.

2,4,6S	HSO_3 | 4 ΔUA1-3GalNAc |2 |6 HSO_3 HSO_3	7×10^{-12}
IVA	ΔUA1-4GlcNAc	5×10^{-7}
Set 5		
IIA	ΔUA1-4GlcNAc |6 HSO_3	7×10^{-10}
IS	HSO_3 |2 ΔUA1-4GlcN |2 |6 HSO_3 HSO_3	2×10^{-10}

	IC50 ng/ml
Bovine Submaxillary Mucin	
Heparin	0.01
Chondroitin Sulphate A	1
Chondroitin Sulphate B	7
Chondroitin Sulphate C	2
Keratan Sulphate	20
	>100

REFERENCES

1. Feizi, T. Nature **314**, 53-57 (1985).
2. Hakomori, S. Cancer Res. **45**, 2405-2414 (1985).
3. Feizi, T. et al., Biochem.J. **245**, 1-11 (1987).
4. Feizi, T. In Carbohydrate Recognition in Cellular Function (Bock G. & Harnett S. eds.) Wiley, Chichester (Ciba Foundation Symposium 145) pp. 62-79 (1989).
5. Drickamer, K. Curr. Opin. Struct. Biol. **3**, 393-400 (1993).
6. Feizi, T. et al., Curr. Opin. Struct. Biol. **3**, 701-710 (1993).
7. Bevilacqua, M. et al., J. Clin. Invest. **91**, 379-387 (1993).
8. Lasky, L. A. et al. Cell **56**, 1045-1055 (1989).
9. McEver, R. P. J. Cell Biochem. **45**, 156-161 (1991).
10. Chambers, W. H. et al., Glycobiology **3**, 9-14 (1993).
11. Yokoyama, W. M. Curr. Opin. Immunol. **5**, 67-73 (1993)
12. Trinchieri, G. Adv. Immunol. **47**, 187-376 (1989).
13. Giorda, R. et al., J. Immunol. **147**, 1701-1708 (1991).
14. Rudd, C. E. et al. Biochim. Biophys. Acta **1155**, 239-266 (1993).
15. Giorda, R. et al., Science **249**, 1298-1300 (1990).
16. Ryan, J. C. et al., J. Immunol. **149**, 1631-1635 (1992).
17. Yokoyama, W. M. et al., Annu. Rev. Immunol. **11**, 613-635 (1993).
18. Chambers, W. H. et al. J. Exp. Med. **169**, 1373-1389 (1989).
19. Ryan, J. C. et al., J. Immunol. **147**, 3244-3250 (1991).

20. Hudig, D. et al., Curr. Opin. Immunol. 5, 90-96 (1993).

21. Bezouška, K. et al., J. Biol. Chem., in press.

22. Kjellen, L. et al., Annu. Rev. Biochem. **60**, 443-475 (1991).

23. Scudder, P. et al. Eur. J. Biochem. **157**, 365-373 (1986).

24. Karlhofer, F. M. et al., Nature **358**, 66-70 (1992).

25. Ryan, J.C. et al., Natural Immunity and Cell Growth Regulation **11**, 279 (Abstr.) (1992).

26. McCoy, J.P.Jr. et al., Glycobiology **1**, 321-328 (1991).

27. Ando, I. et al., Int. J. Cancer **40**, 12-17 (1987).

28. Grayson, G. et al., Cell. Immunol.**139**, 18-29 (1992).

29. Yamamoto, H. et al., Cell. Immunol. **96**, 409-417 (1985).

30. Young, W.M. Jr. et al., J. Immunol. **126**, 1-6 (1981).

31. Forbes, J.T. et al., Proc. Natl. Acad. Sci. USA **78**, 5797-5801 (1981).

32. Chambers, W. H. et al., J. Immunol. **137**, 1469-1474(1986).

33. Haubeck, H.-D. et al., J. Immunol. **134**, 65-69 (1985).

34. von Figura, K. et al., Annu. Rev. Biochem. **55**, 167-193 (1986).

35. Young, W. W. Jr. et al., J. Immunol. **124**, 199-201 (1980).

36. Chou, D.K.H. et al., J. Biol. Chem. **261**, 11717-11725 (1986).

37. Ohmori, K. et al. Blood **74**, 255-261 (1989).

38. Stoll, M.S. et al., Biochem. J. **256**, 661-664 (1988).

39. Gooi, H.C. et al. J. Biol. Chem. **260**, 13218-1322 (1985).

40. Larkin, M. et al. J. Biol. Chem. **267**, 13661-13668 (1992).

41. Yuen, C.-T. et al. J. Biol. Chem. **269**, 1595-1598 (1994).

42. Loveless, R. W. et al., EMBO J. **11**, 813-819 (1992).

43. Schwarzmann, G. et al., Meth. Enzymol. **138**, 319-341 (1987).

44. Feizi, T. et al., Meth. Enzymol. **230**, 484-519 (1993).

45. Vujanovic, N. L., et al., J. Exp. Med. **167**, 15-29 (1988).

46. Ward, J. M. et al., Pathol. **111**, 1-10 (1983).

47. Brunner, K. T. et al., Immunology **14**, 181-196 (1968).

48. Feizi, T., et al., exp. Med. **149**, 975-980 (1979).

49. Chused et al *Cytometry* **8**, 396-404 (1987).

50. Radcliff et al., *J. Immunol. Meth.* **139** 281-292 (1991).

51. Zanetti M. et al, *EMBO J.* **12**, 4375-4384 (1993).

52. Quillet-Mary et al, *Int. J. Cancer* **47**, 473-479 (1991).

53. Roellinger et al., *Scand J. Immunol* **25**, 507-515 (1987).

54. Hamann et al., *J. Immunol.* **150**, 4920-4927 (1993).

55. López-Cabrera et al., *J. Exp. Med.* **178**, 537-547 (1993).

56. Ziegler et al., *Eur. J. Immunol* **23**, 1643-1648 (1993).

57. Testi et al., *Immunology Today* **15**, 479-483 (1994).

58. Testi et al., *J. Immunol*, **143**, 1123-1128 (1989).

59. Nakamura et al., *J. Exp. Med.* **169**, 677-689 (1989).

60. Santis et al., *Eur. J. Immunol* **22**, 1253-1259 (1992).

61. Isler et al., *Eur. Cytokine Netw.* **4**, 15-23 (1993).

62. Manié et al., *Eur. Cytokine Netw.* **4**, 7-13 (1993).

63. Trinchieri G. *J. Exp. Med.* **180**, 417-421 (1994).

64. Feizi et al., *Glycobiology*, **4**, 106-109 (1994).

65. Romagnai, *Immunology Today*, **13**, 379 (1992).

66. Lanier et al., *J. Exp. Med.* **169**, 2233-2238 (1989).

67. Zhang et al., *J. Biol. Chem*, **269**, 19295-19299 (1994).

68. Abdoullah, K.M., et al, *Infect. Immun.* **60**, 56-62 (1992).

69. Bazil, V. et al., *Eur. J. Immunol* **16**, 1583-1589 (1986).

70. Bezouška, K. et al., *Mol. Immunol* **29** 1437-1446; *J. Biol. Chem.* **269**, 16945-16952 (1994); *Nature*, **372**, 150-157.

71. Borrego, F. et al., *Eur. J. Immunol.* **23**, 1039-1043 (1993).

72. Brummer, K.T. et al., *Immunology* **14**, 181-196 (1968).

73. Cerwenka, A. et al., *J. Immunol*, **153**, 4367-4377 (1994).

74. Correa-Freire, M.C. et al., *J. Immunol.* **132**, 69-75 (1984).

75. Eischen, C.M. et al., *J. Immunol.* **153**, 1947-1954 (1994).

76. Feizi, T. et al., *Biochem. J.* **173**, 245-254 (1978).

77. Folks, T.M. et al., *Science*, **242** 919-922 (1988).

78. Hamann, J. et al., *J. Immunol.* **150**, 4920-4927 (1993).

79. Harlow, E. et al., *E. Harlow and D. Lane eds.* (1988).

80. Horejsi, V. et al., *Folia Biol.* **34**, 23-34 (1988).

81. Isler, P. et al., *Eur. Cytokine Netw.* **4**, 15-23 (1993).

82. Karlhofer, F.M. et al., *J. Immunol.* **146**, 3662-3673 (1991).

83. Kojima, H. et al., *Immunity* **1**, 357-364 (1994).

84. Laemmli, *Nature* **227**, 680-685 (1970).

85. Robinson, P.J. *Immunological Methods* Vol. III (eds Lefkovitz, I.& Pernis, B.) 125-132 (1979).

86. Roussel. M.F. et al., *Proc. Natl. Adac. Sci.* **85**, 5903-5907 (1988).

87. Sambrook, J. et al., *J. Sambrook, E.F. Fritsch and T. Maniatis eds* (1989).

88. Ebel, F. et al., *Biochemistry* **31**, 12190-12197 (1992).

89. Schwarting, R. et al., *Oxford University Press*, Vol. IV, pp 428-432 (1989).

90. Scudder, P. et al., *Eur. J. Biochem.* **157**, 365-373 (1986).

91. Sgonc, R. et al., *Trends. Genet.* **10**, 41-42 (1994).

92. Stefanová, I. *Folia Biol.* **34**, 255-265 (1988).

93. Stefanová, I., *Immunogenetics.* **29**, 402-404 (1989).

94. Timonen, T. et al., *J. Exp. Med.* **153**, 569-582 (1981).

95. Wang, P.L. et al., *J. Immunol.* **148**, 2600-2608 (1992).

96. Yokoyama, W.M. et al., *Annu. Rev. Immunol* **11**, 613-635 (1993).

97. Yokoyama, W.M. et al., *J. Immunol* **145**, 2353-2358 (1990).

98. Yonehara, S. *J. Exp. Med.* **169**, 1747-1756 (1989).

99. Ziegler, S.F. et al., *J. Immunol.* **152**, 1228-1236 (1994).

100. Ziegler, S.F. et al., *Stem Cells*, **12**, 456-465 (1994).

CLAIMS

1. A method of modulating activity of effector cells of the immune system, comprising contacting the cells with an oligosaccharide which comprises a glycosaminoglycan oligosaccharide a sulphated ganglioside other than sulphatide, a 6-sialyl hexose or 3-O-sulphated uronic acid.

2. A method according to claim 1 wherein the oligosaccharide is a keratan sulphate, a chondroitin sulphate or a heparin sulphate.

3. A method according to claim 1 or claim 2 wherein the oligosaccharide is a disaccharide or a tetrasaccharide.

4. A method according to claim 1 wherein the oligosaccharide is selected from the group consisting of: K6; Chon OS, 6S, 2,6S and 2,4,6S; and Hep IVA, IIA and IS, HNK-1, 6SN, 6S2, 6SLN, S2 and SN.

5. A method of modulating activity of effector cells which are Natural Killer (NK) cells, comprising contacting the cells with a oligosaccharide which comprises a glycosaminoglycan oligosaccharide, sulphatide, a sulphated ganglioside other than sulphatide, a 6-sialyl hexose or 3-O-sulphated uronic acid, tetramannose phosphate, pentamannose phosphate,

Sialyl- or sulphated- Le^a or Le^x; HNK-1; HNK-3-5-uronic acid.

6. A method according to claim 5 wherein the oligosaccharide is selected from keratan sulphates, such as K6; S2; SN; 6ST; Chondroitin sulphates, such as ChonSO_3 OS/6S, 2,6S and 2,4,6S; Heparin oligosaccharides, such as HepIVA, IIA, IS and IS2.

7. A method according to any one of the preceding claims wherein the oligosaccharide is free and effector cell function directed at target cells is inhibited.

8. A method according to any one of claims 1 to 6 wherein the oligosaccharide is clustered and effector cell function directed at target cells is augmented.

9. A method of increasing activity of effector cells of the immune system, comprising contacting the cells with clustered monosaccharide or oligosaccharide.

10. A method according to claim 9 wherein the effector cells are Natural Killer (NK) cells.

11. A method according to claim 10 wherein the monosaccharide or oligosaccharide is a ligand of NKR-P1.

12. A method according to any one of claims 9 to 11 wherein the monosaccharide or oligosaccharide is a ligand of CD69.

13. A method according to claim 11 or claim 12 wherein the monosaccharide or oligosaccharide has the structure:

A(v,w)-B(1,x)-C(1-y)--D

wherein:

A is selected from: hydrogen; a hexose that may be galactose or mannose and may be substituted with one or more charged moieties; substituted sialic acid; an aliphatic chain with one or more branched moieties; saturated or unsaturated uronic acid that may be substituted with one or more charged moieties; N-acetylglucosamine that may be substituted with one or more charged moieties; and N-acetylgalactosamine that may be substituted with one or more charged moieties;

B may be absent or selected from: uronic acid that may be substituted with one or more charged moieties; galactose; N-acetylglucosamine that may be substituted with one or more charged moieties, galactose or fucose; N-acetylgalactosamine that may be substituted with one or more charged moieties; an aliphatic chain that may be substituted with one or more charged moieties; and an oligosaccharide chain or 3 or 4 mannose residues;

C may be absent or selected from: uronic acid

that may be substituted with one or more charged moieties; galactose; glucose; N-acetylglucosamine that may be substituted with one or more charged moieties; N-acetylgalactosamine that may be substituted with one or more charged moieties; and an aliphatic chain that may be substituted with one or more charged moieties;

D may be absent or selected from: one or more repeats of B and/or C; remaining structural components of N-linked bi-, tri- or tetra-antennary oligosaccharides; O-glycosidic oligosaccharides and glycosaminoglycans; and other sequences serving to support or present the ligand;

the parenthetical lower case letters represent the position of bonded carbons of the indicated carbohydrates where v = 1 or 2, w = 2, 3, 4 or 6, x = 2, 3 or 4, y = 2, 3, 4 or 6.

when A is a substituted sialic acid the substitutions may be N-acetyl or O-acetyl;

when B or C is a substituted aliphatic chain, the substitutions may be selected from hydroxyl(s), acetyl amino ($NH.COCH_3$) and charged moiety(ies); and

charged moieties may be selected from the group consisting of sulphate, phosphate and carboxylic, e.g. sialic, acid (other charged groups may be employed).

14. A method according to claim 13 wherein the monosaccharide or oligosaccharide comprises: α2-3 linked sialic acid, 3-O-sulphation, 3-O-sulphated

galactose, 3-, 4- or 6- O-sulphated N-acetyl hexosamine, N-sulphated hexosamine, uronic acid-substituted N-acetylhexosamine or hexosamine, 2- or 3-O-sulphated uronic acid, α1-3-linked fucose, α1-4-linked fucose, α2-6-linked sialic acid, α2-6-linked sialic acid on galactose or on N-acetylhexose.

15. A method according to claim 11 or claim 12 wherein the oligosaccharide is selected from: glycosaminoglycan oligosaccharide, sulphatide, a sulphated ganglioside other than sulphatide, a 6-sialyl hexose or 3-O-sulphated uronic acid, tetramannose phosphate, pentamannose phosphate, Sialyl- or sulphated- Le^a or Le^x; HNK-1; HNK-3-5-uronic acid, keratan sulphates, such as K6; S2; SN; 6ST; Chondroitin sulphates, such as ChonSO_3 OS/6S, 2,6S and 2,4,6S; and Heparin oligosaccharides, such as HepIVA, IIA, IS and IS2.

16. A method according to any one of claims 1 to 15 wherein cells at which effector function is directed ("target cells") are treated with the monosaccharide or oligosaccharide and then treated with the effector cells.

17. A method according to claim 16 wherein the treatment with monosaccharide or oligosaccharide is targetted to the target cells.

18. A method according to claim 16 wherein the treatment with the effector cells is targetted to the target cells.

19. A method according to claim 17 or claim 18 wherein the targetting employs an antibody or an antibody fragment able to bind antigen.

20. A method according to any one of claims 1 to 15 wherein cells at which effector function is directed ("target cells") are treated with effector cells and then treated with the monosaccharide or oligosaccharide.

21. A method according to claim 20 wherein the treatment with monosaccharide or oligosaccharide is targeted to the target cells.

22. A method according to claim 20 wherein the treatment with the effector cells is targeted to the target cells.

23. A method according to claim 21 or claim 22 wherein the targetting employs an antibody or an antibody fragment able to bind antigen.

24. A method according to any one of claims 8 to 15 wherein the monosaccharide or oligosaccharide is

clustered on a liposome.

25. A method according to claim 24 wherein the liposome comprises a first member of a specific binding pair (sbp member) able to bind a complementary second member of the specific binding pair.

26. A method according to claim 25 wherein the second sbp member is on the surface of cells at which the effector function is directed ("target cells").

27. A method according to claim 25 or claim 26 wherein the first sbp member is an antibody or fragment thereof able to bind a complementary second sbp member.

28. A method according to any one of claims 8 to 15 wherein the monosaccharide or oligosaccharide is clustered on a sequence of amino acids.

29. A method according to claim 28 wherein the sequence of amino acids is part of a molecule comprising a first member of a specific binding pair (sbp member) able to bind a complementary second member of the specific binding pair.

30. A method according to claim 29 wherein the second sbp member is on the surface of cells at which the effector function is directed ("target cells").

31. A method according to claim 29 or claim 30 wherein the first sbp member is an antibody or fragment thereof able to bind a complementary second sbp member.

32. A method according to any one of the preceding claims wherein the activity modulated is anti-proliferative activity, cytotoxicity and/or cytokine secretion.

33. A method according to claim 32 wherein the cytotoxicity comprises stimulation of apoptosis in target cells.

34. A method according to any one of the preceding claims wherein effector function is directed at target cells which are tumour cells.

35. A method according to any one of the preceding claims wherein the cells are contacted with the monosaccharide or oligosaccharide *in vitro*.

36. A method according to any one claims 1 to 34 wherein the cells are contacted with the monosaccharide or oligosaccharide *in vivo*.

37. Use of an oligosaccharide which comprises a glycosaminoglycan oligosaccharide a sulphated ganglioside other than sulphatide, a 6-sialyl hexose or

3-O-sulphated uronic acid, in the manufacture of a composition for modulating activity of effector cells of the immune system.

38. Use according to claim 37 wherein the oligosaccharide is a keratan sulphate, a chondroitin sulphate or a heparin sulphate.

39. Use according to claim 37 or claim 38 wherein the oligosaccharide is a disaccharide or a tetrasaccharide.

40. Use according to claim 37 wherein the oligosaccharide is selected from the group consisting of: K6; Chon OS, 6S, 2,6S and 2,4,6S; and Hep IVA, IIA and IS, HNK-1, 6SN, 6S2, 6SLN, S2 and SN.

41. Use of an oligosaccharide which comprises a glycosaminoglycan oligosaccharide, sulphatide, a sulphated ganglioside other than sulphatide, a 6-sialyl hexose or 3-O-sulphated uronic acid, tetramannose phosphate, pentamannose phosphate, Sialyl- or sulphated- Le^a or Le^x; HNK-1; HNK-3-5-uronic acid, in the manufacture of a composition for modulating activity of effector cells which are Natural Killer (NK) cells.

42. Use according to claim 41 wherein the oligosaccharide is selected from keratan sulphates,

such as K6; S2; SN; 6ST; Chondroitin sulphates, such as ChonSO_3 OS/6S, 2,6S and 2,4,6S; Heparin oligosaccharides, such as HepIVA, IIA, IS and IS2.

43. Use according to claim 42 wherein the oligosaccharide is free and effector cell function directed at target cells is inhibited.

44. Use according to claim 43 wherein the oligosaccharide is clustered and effector cell function directed at target cells is augmented.

45. Use of clustered monosaccharide or oligosaccharide in the manufacture of a composition for increasing activity of effector cells of the immune system.

46. Use according to claim 45 wherein the effector cells are Natural Killer (NK) cells.

47. Use according to claim 46 wherein the monosaccharide or oligosaccharide is a ligand of NKR-P1.

48. Use according to claim 46 or claim 47 wherein the monosaccharide or oligosaccharide is a ligand of CD69.

49. Use according to claim 47 or claim 48 wherein the

monosaccharide or oligosaccharide has the structure:

A(v,w)-B(1,x)-C(1-y)--D

wherein:

A is selected from: hydrogen; a hexose that may be galactose or mannose and may be substituted with one or more charged moieties; substituted sialic acid; an aliphatic chain with one or more branched moieties; saturated or unsaturated uronic acid that may be substituted with one or more charged moieties; N-acetylglucosamine that may be substituted with one or more charged moieties; and N-acetylgalactosamine that may be substituted with one or more charged moieties;

B may be absent or selected from: uronic acid that may be substituted with one or more charged moieties; galactose; N-acetylglucosamine that may be substituted with one or more charged moieties, galactose or fucose; N-acetylgalactosamine that may be substituted with one or more charged moieties; an aliphatic chain that may be substituted with one or more charged moieties; and an oligosaccharide chain or 3 or 4 mannose residues;

C may be absent or selected from: uronic acid that may be substituted with one or more charged moieties; galactose; glucose; N-acetylglucosamine that may be substituted with one or more charged moieties; N-acetylgalactosamine that may be substituted with one or more charged moieties; and an aliphatic chain that may be substituted with one or more charged moieties;

D may be absent or selected from the group consisting of one or more repeats of B and/or C; remaining structural components of N-linked bi-, tri- or tetra-antennary oligosaccharides; O-glycosidic oligosaccharides and glycosaminoglycans; and other sequences serving to support or present the ligand;

the parenthetical lower case letters represent the position of bonded carbons of the indicated carbohydrates where v = 1 or 2, w = 2, 3, 4 or 6, x = 2, 3 or 4, y = 2, 3, 4 or 6.

when A is a substituted sialic acid the substitutions may be N-acetyl or O-acetyl;

when B or C is a substituted aliphatic chain, the substitutions may be selected from hydroxyl(s), acetyl amino ($NH.COCH_3$) and charged moiety(ies); and

charged moieties may be selected from sulphate, phosphate and carboxylic, e.g. sialic, acid.

50. Use according to claim 49 wherein the monosaccharide or oligosaccharide comprises: α2-3 linked sialic acid, 3-O-sulphation, 3-O-sulphated galactose, 3-, 4- or 6- O-sulphated N-acetyl hexosamine, N-sulphated hexosamine, uronic acid-substituted N-acetylhexosamine or hexosamine, 2- or 3-O-sulphated uronic acid, α1-3-linked fucose, α1-4-linked fucose, α2-6-linked sialic acid, α2-6-linked sialic acid on galactose or on N-acetylhexose.

51. Use according to claim 49 or claim 50 wherein the oligosaccharide is selected from: glycosaminoglycan oligosaccharide, sulphatide, a sulphated ganglioside other than sulphatide, a 6-sialyl hexose or 3-O-sulphated uronic acid, tetramannose phosphate, pentamannose phosphate, Sialyl- or sulphated- Le^a or Le^x; HNK-1; HNK-3-5-uronic acid, keratan sulphates, such as K6; S2; SN; 6ST; Chondroitin sulphates, such as ChonSO_3 OS/6S, 2,6S and 2,4,6S; and Heparin oligosaccharides, such as HepIVA, IIA, IS and IS2.

52. Use according to any one of claims 37 to 51 wherein the composition is for treatment of cells at which effector function is directed ("target cells") prior to treatment of the target cells with the effector cells.

53. Use according to claim 52 wherein said treatment with the composition is targetted to the target cells.

54. Use according to claim 52 wherein said treatment with the effector cells is targetted to the target cells.

55. Use according to claim 53 or claim 54 wherein the targetting employs an antibody or an antibody fragment able to bind antigen.

56. Use according to any one of claims 37 to 51 wherein the composition is for treatment of cells at which effector function is directed ("target cells") following treatment of the target cells with said effector cells.

57. Use according to claim 56 wherein said treatment with the composition is targeted to the target cells.

58. Use according to claim 56 wherein said treatment with the cells of the immune system is targeted to the target cells.

59. Use according to claim 57 or claim 58 wherein the targetting employs an antibody or an antibody fragment able to bind antigen.

60. Use according to any one of claims 44 to 51 wherein the monosaccharide or oligosaccharide is clustered on a liposome.

61. Use according to claim 60 wherein the liposome comprises a first member of a specific binding pair (sbp member) able to bind a complementary second member of the specific binding pair.

62. Use according to claim 61 wherein the second sbp member is on the surface of cells at which the effector

function is directed ("target cells").

63. Use according to claim 61 or claim 62 wherein the first sbp member is an antibody or fragment thereof able to bind a complementary second sbp member.

64. Use according to any one of claims 44 to 51 wherein the monosaccharide or oligosaccharide is clustered on a sequence of amino acids.

65. Use according to claim 64 wherein the sequence of amino acids is part of a molecule comprising a first member of a specific binding pair (sbp member) able to bind a complementary second member of the specific binding pair.

66. Use according to claim 65 wherein the second sbp member is on the surface of cells at which the effector function is directed ("target cells").

67. Use according to claim 65 or claim 66 wherein the first sbp member is an antibody or fragment thereof able to bind a complementary second sbp member.

68. Use according to any one of claims 37 to 67 wherein the activity modulated is anti-proliferative activity, cytotoxicity and/or cytokine secretion.

69. Use according to claim 68 wherein the cytotoxicity comprises stimulation of apoptosis in target cells.

70. Use according to any one claims 37 to 69 wherein effector function is directed at target cells which are tumour cells.

71. A composition comprising an oligosaccharide comprising a glycosaminoglycan oligosaccharide, sulphatide, a sulphated ganglioside other than sulphatide, a 6-sialyl hexose or 3-O-sulphated uronic acid, tetramannose phosphate, pentamannose phosphate, Sialyl- or sulphated- Le^a or Le^x; HNK-1; HNK-3-5-uronic acid for use in a method of treatment of a mammal.

72. A composition according to claim 71 wherein the treatment comprises modulation of the activity of effector cells of the immune system.

73. A composition according to claim 72 wherein the effector cells are Natural Killer (NK) cells.

74. A composition according to any one of claims 71 to 73 further comprising a pharmaceutically acceptable excipient or carrier.

75. A method for obtaining a ligand of NKR-P1 and/or

CD69, comprising screening molecules for ability to bind NKR-P1 and/or CD69, or a fragment of NKR-P1 and/or CD69, and selecting a molecule which has said ability.

76. A method according to claim 75 wherein said screening involves binding competition with a known ligand of NKR-P1 and/or CD69.

77. A method according to claim 75 or claim 76 wherein the NKR-P1 and/or CD69, or fragment of NKR-P1 and/or CD69, is labelled.

78. A method according to any one of claims 75 to 77 wherein the selected molecule (ligand) is a monosaccharide or oligosaccharide.

79. A method according to claim 78 wherein the monosaccharide or oligosaccharide is part of a glycopeptide, glycoprotein, glycolipid or proteoglycan.

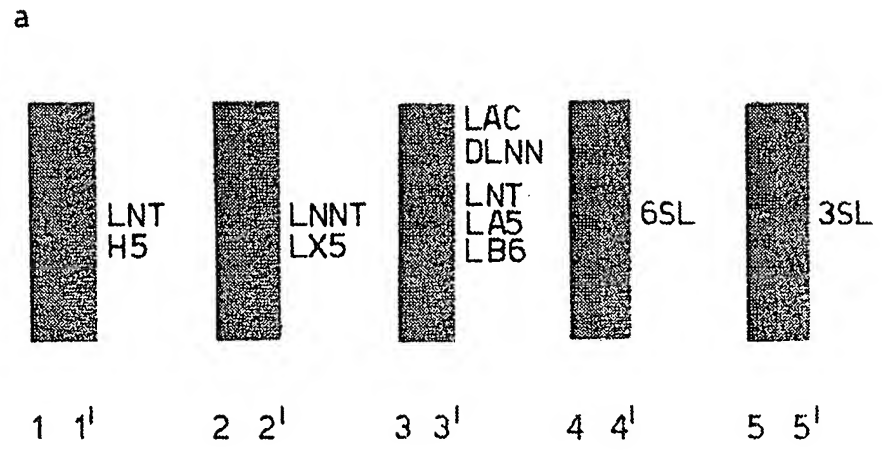
80. A method which comprises, following selection of a ligand of NKR-P1 and/or CD69 using a method according to any one of claims 75 to 79, modulation of activity of effector cells of the immune system comprising contacting the cells with the ligand.

81. A method which comprises, following selection of a ligand of NKR-P1 and/or CD69 using a method according

to any one of claims 75 to 79, use of the ligand in the manufacture of a composition for modulating activity of effector cells of the immune system.

82. A method according to claim 80 or claim 81 wherein the effector cells are NK cells.

Fig.1.


a
LNT
H5
LNNT
LX5
LAC
DLNN
LNT
LA5
LB6
6SL
3SL
1 1'
2 2'
3 3'
4 4'
5 5'

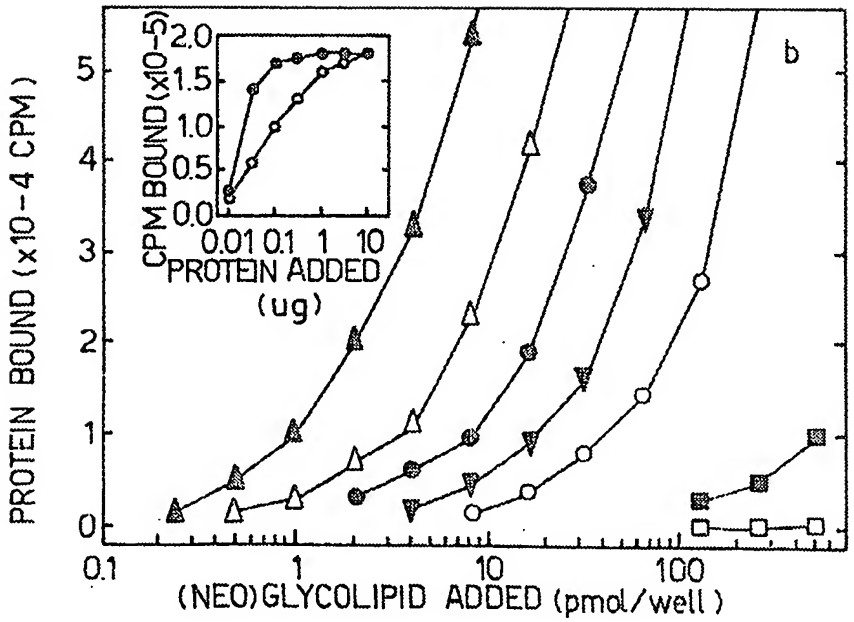

b
PROTEIN BOUND (x10-4 CPM)
(NEO)GLYCOLIPID ADDED (pmol/well)
CPM BOUND (x10-5)
PROTEIN ADDED (ug)

Fig. 1.

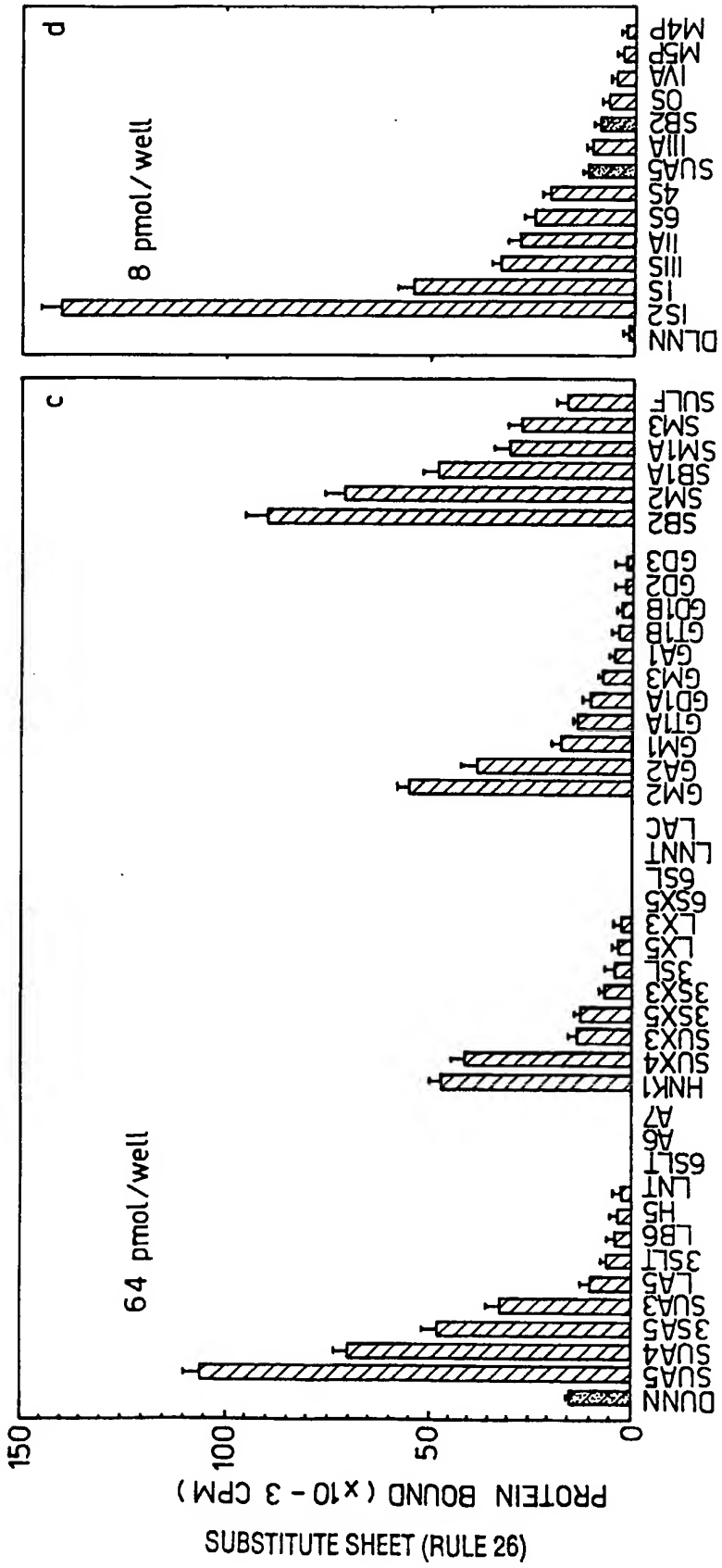

c
64 pmol/well
d
8 pmol/well
DUNN SUA5 SUA4 3SA5 SUA3 LA5 3SL1 LB6 H5 LNT1 6SL1 A6 A7 HNK1 SUX4 SUX3 3SX5 3SX3 3SL LX5 LX3 6SX5 6SL LNNT LAC GM2 GA2 GM1 GT1A GD1A GM3 GA1 GT1B GD1B GD2 GD3 SB2 SM2 SB1A SM1A SM3 SULF
DLNN IS2 IS IIIS IIA 6S 4S SUA5 IIIA SB2 OS IVA M5P M4P
150 100 50 0
PROTEIN BOUND (x10 - 3 CPM)

Fig.2.

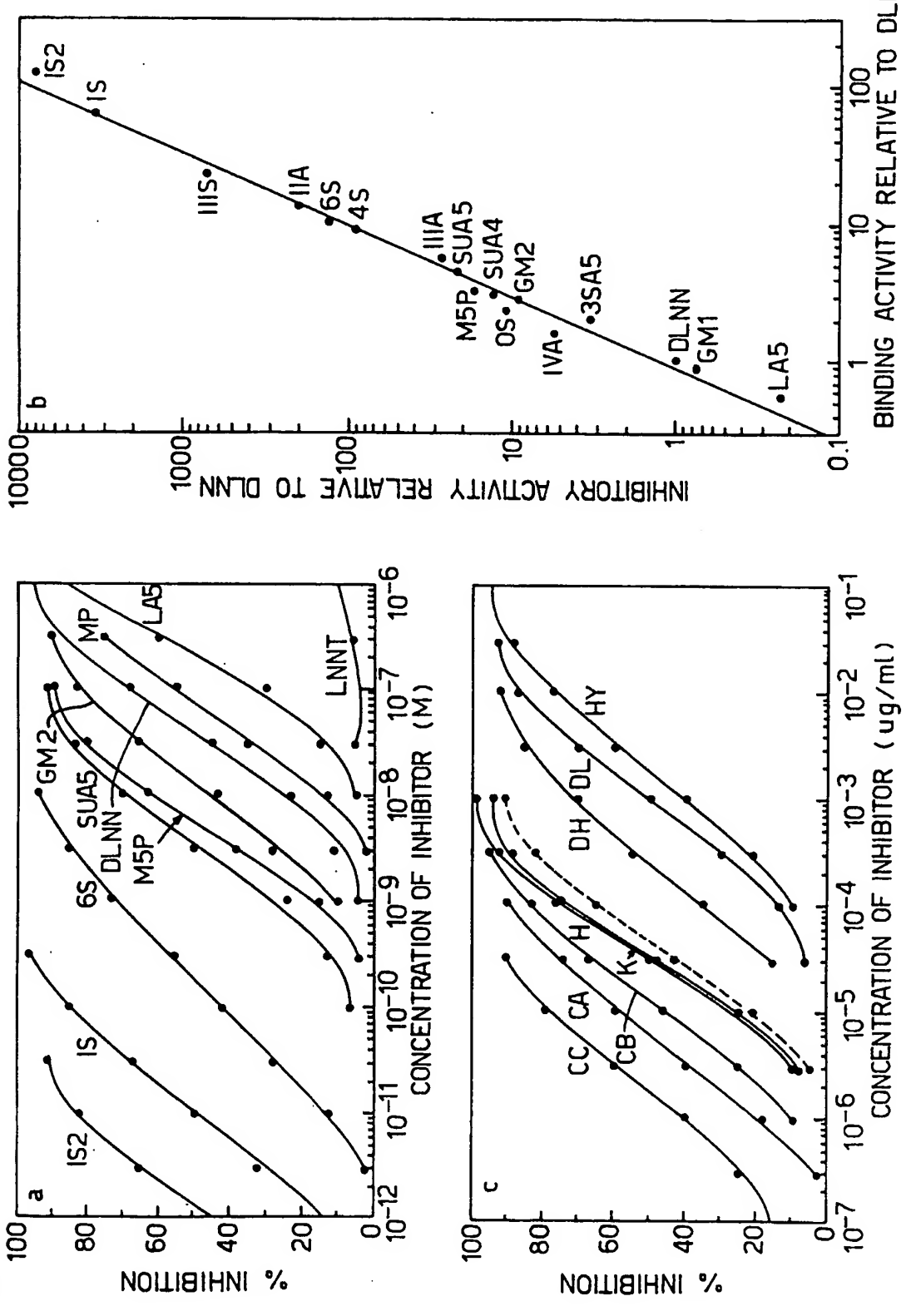
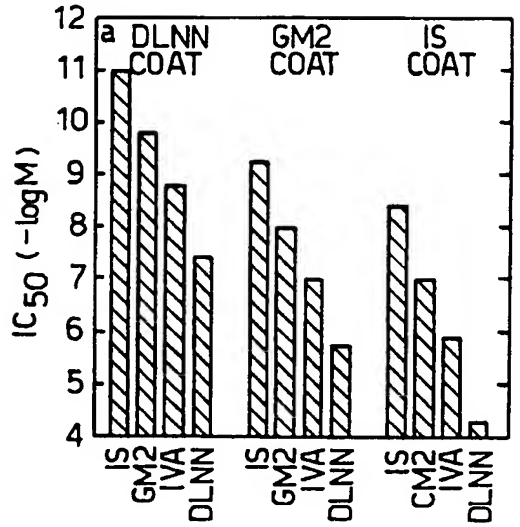

a
IS2
IS
6S
DLNN
SUA5
GM2
M5P
MP
LA5
LNNT
% INHIBITION
CONCENTRATION OF INHIBITOR (M)
b
IS2
IS
IIIS
IIA
6S
4S
IIIA
SUA5
SUA4
M5P
OS
GM2
IVA
3SA5
DLNN
GM1
LA5
INHIBITORY ACTIVITY RELATIVE TO DLNN
BINDING ACTIVITY RELATIVE TO DLNN
c
HY
DL
DH
H
K
CA
CC
CB
% INHIBITION
CONCENTRATION OF INHIBITOR (ug/ml)

Fig.3.


a
DLNN COAT
GM2 COAT
IS COAT
IC50 (-logM)
12
11
10
9
8
7
6
5
4
IS
GM2
IVA
DLNN

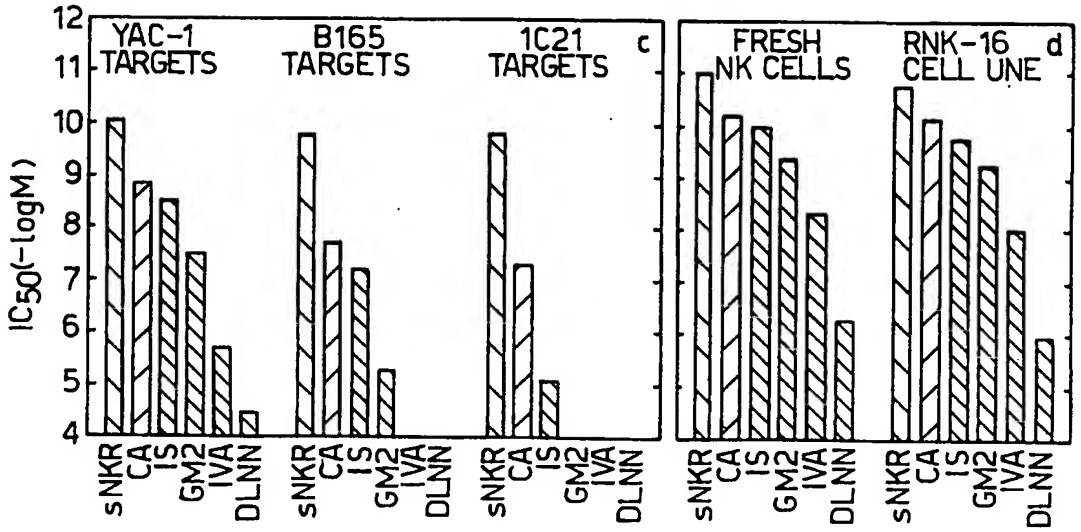
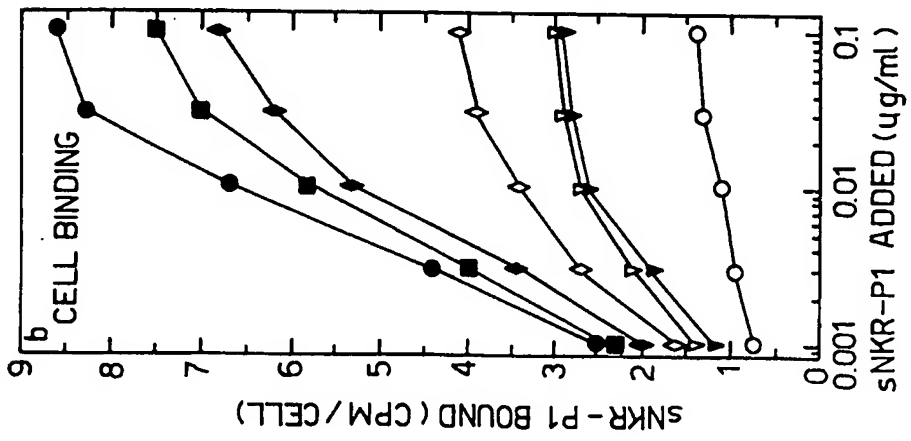

YAC-1 TARGETS
B165 TARGETS
1C21 TARGETS
c
FRESH NK CELLS
RNK-16 CELL LINE
d
IC50(-logM)
12
11
10
9
8
7
6
5
4
sNKR
CA
IS
GM2
IVA
DLNN

Fig.3.


b CELL BINDING
sNKR-P1 BOUND (CPM/CELL)
sNKR-P1 ADDED (µg/ml)
e FRESH NK
f RNK-16
%SPECIFIC LYSIS
E:T
k
IC50 FOR TARGET CELL KILLING (M)
IC50 FOR sNKR-P1 BINDING TO CELLS (M)
g FRESH NK YAC-1
h RNK-16/ YAC-1
i RNK-16/ B16S
j RNK-16/ 1C21
% INHIBITION
CONCENTRATION OF INHIBITOR (M)

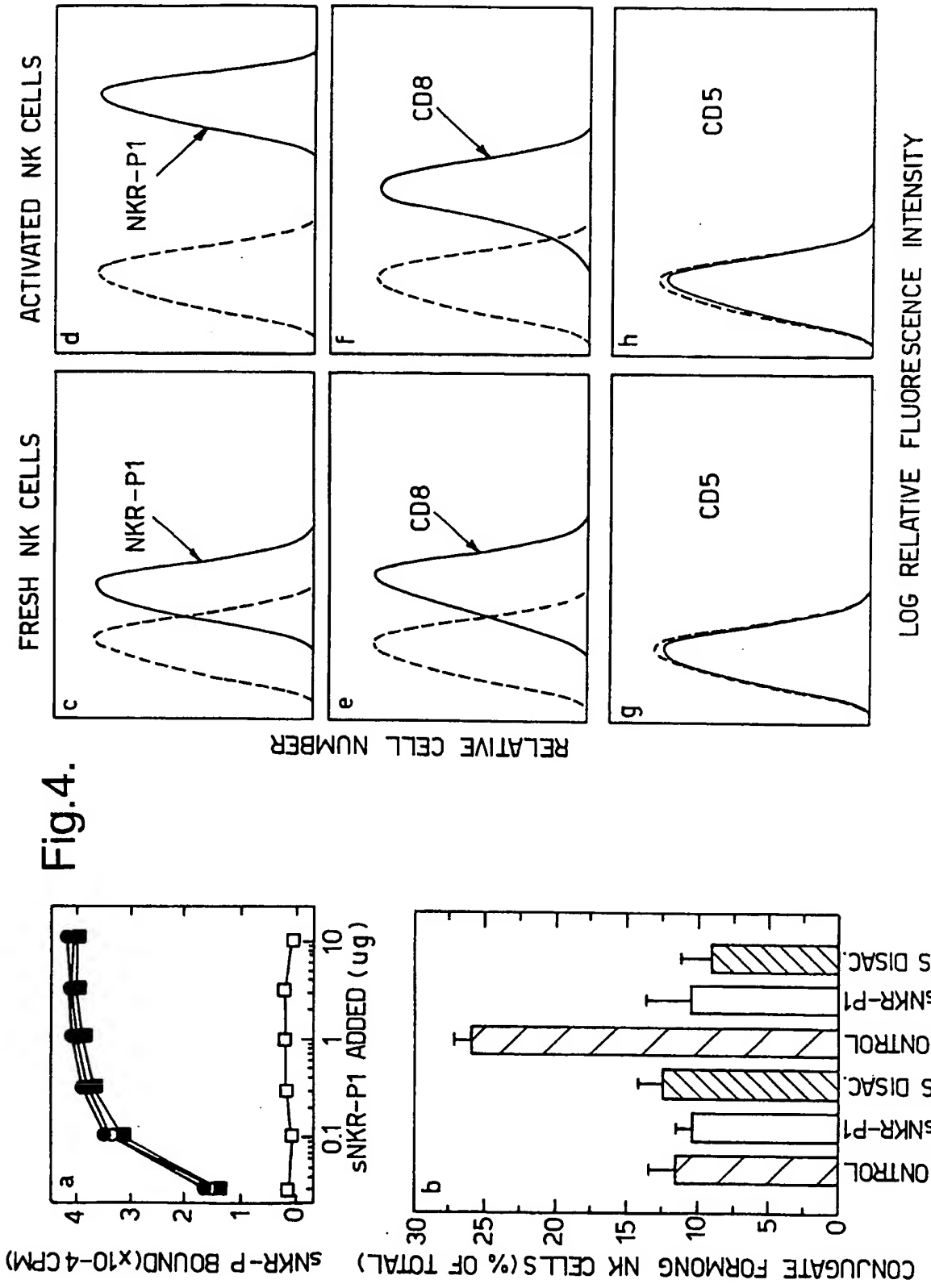

ACTIVATED NK CELLS
FRESH NK CELLS
NKR-P1
CD8
CD5
d
c
f
e
h
g
RELATIVE CELL NUMBER
LOG RELATIVE FLUORESCENCE INTENSITY
a
b
sNKR-P BOUND(x10-4 CPM)
sNKR-P1 ADDED (ug)
CONJUGATE FORMONG NK CELLS (% OF TOTAL)
CONTROL
sNKR-P1
+IS DISAC.
FRESH ACTIVATED

Fig.4.

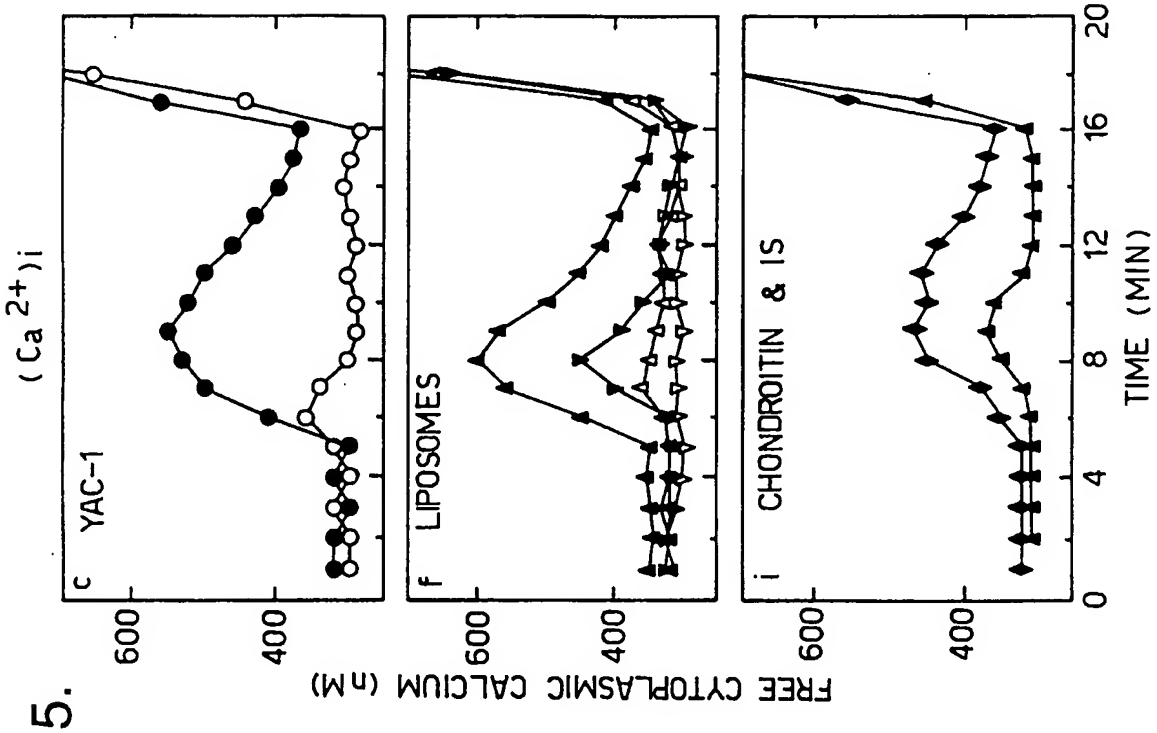

FREE CYTOPLASMIC CALCIUM (nM)
(Ca 2+)i
c YAC-1
f LIPOSOMES
i CHONDROITIN & IS
600
400
0
4
8
12
16
20
TIME (MIN)

Fig.5.

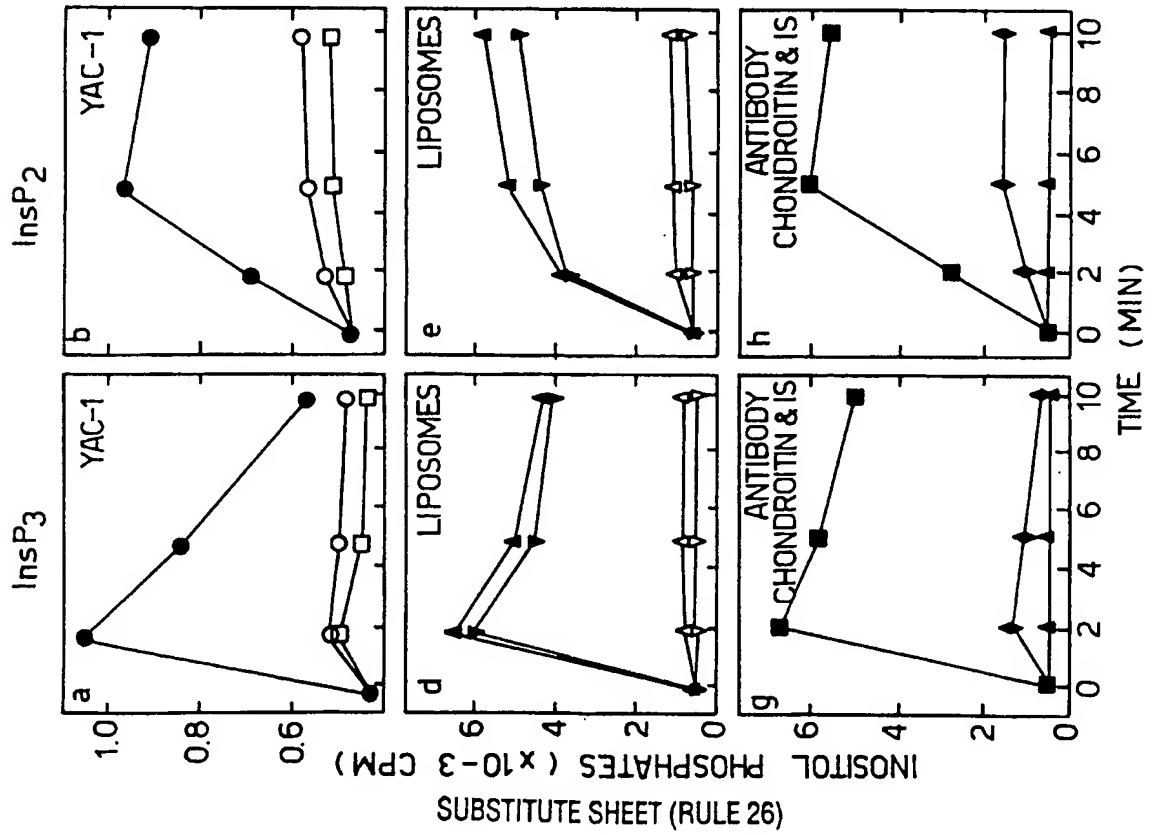

INOSITOL PHOSPHATES (x10-3 CPM)
InsP3
InsP2
a YAC-1
b YAC-1
d LIPOSOMES
e LIPOSOMES
g ANTIBODY CHONDROITIN & IS
h ANTIBODY CHONDROITIN & IS
1.0
0.8
0.6
6
4
2
0
0
2
4
6
8
10
TIME (MIN)

Fig.6.

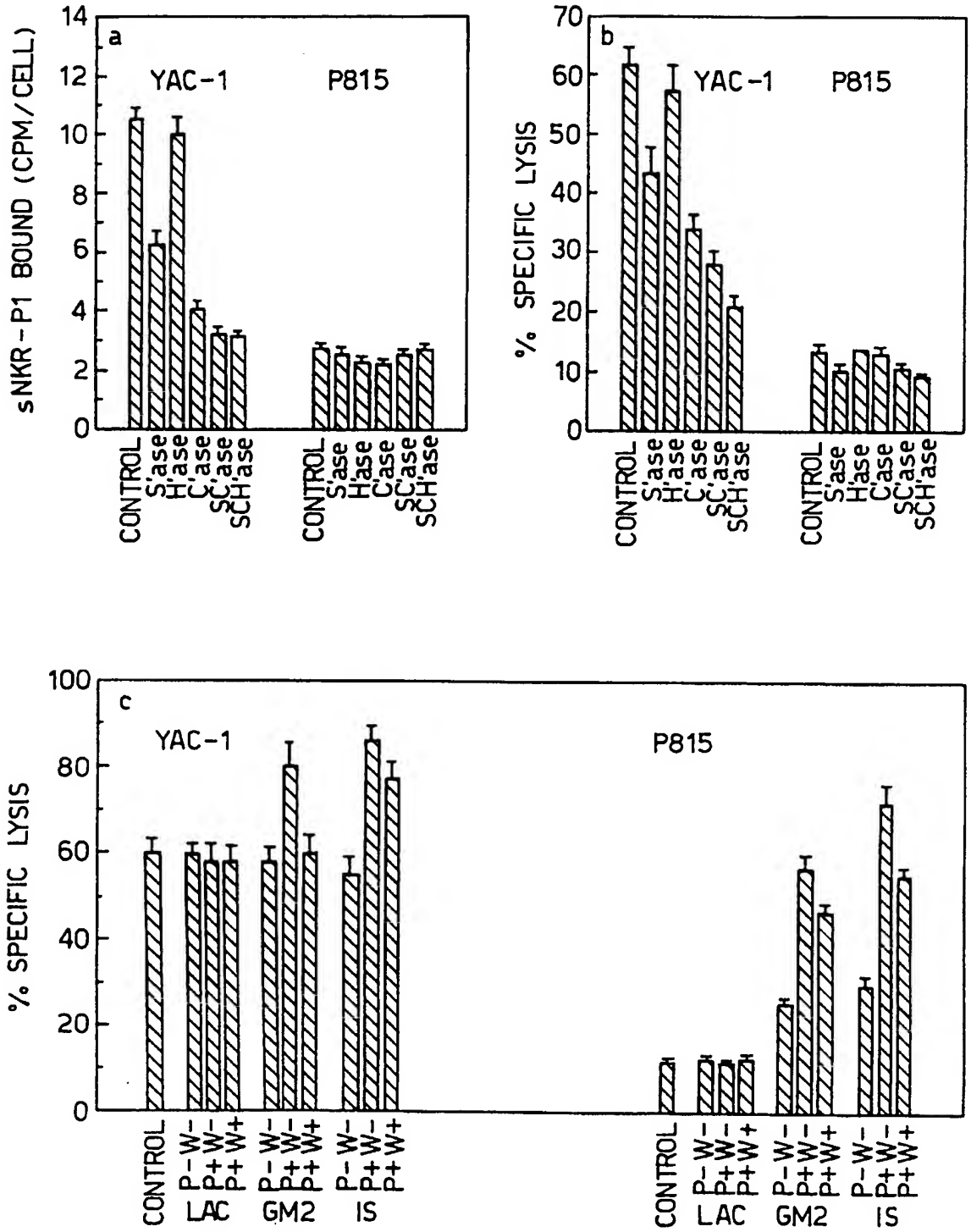

a
YAC-1
P815
sNKR-P1 BOUND (CPM/CELL)
14
12
10
8
6
4
2
0
CONTROL
S'ase
H'ase
C'ase
SC'ase
SCH'ase
b
% SPECIFIC LYSIS
70
60
50
40
30
20
10
0
c
100
80
60
40
20
0
P-W-
P+W-
P+W+
LAC
GM2
IS

Fig.6.

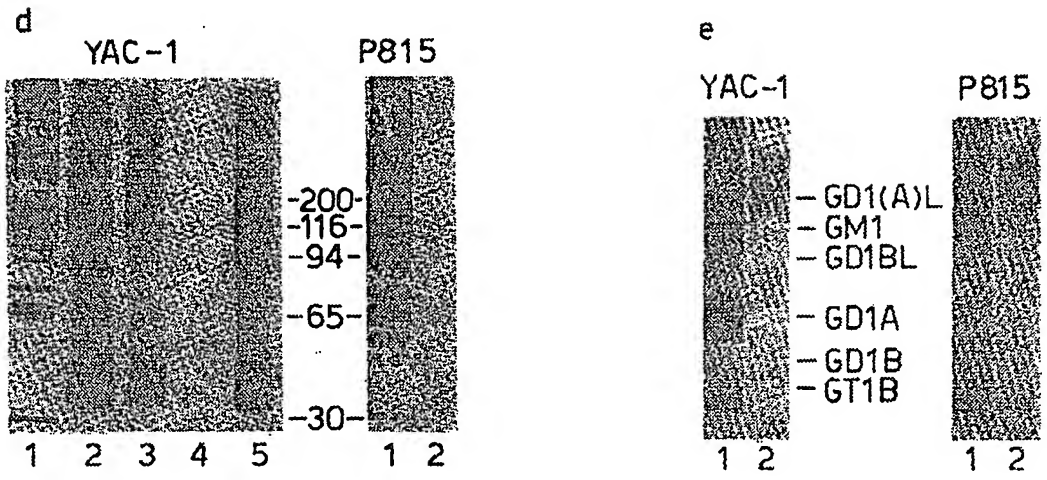

d
YAC-1
P815
-200-
-116-
-94-
-65-
-30-
1 2 3 4 5
1 2
e
YAC-1
P815
GD1(A)L
GM1
GD1BL
GD1A
GD1B
GT1B
1 2
1 2

Fig.9.

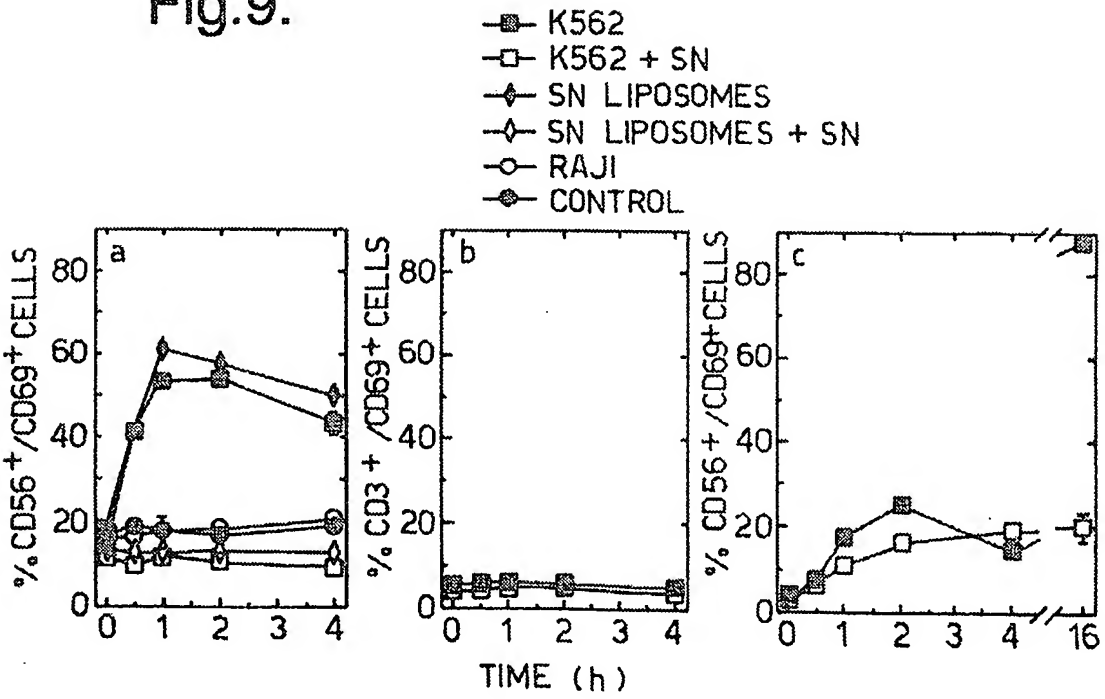

K562
K562 + SN
SN LIPOSOMES
SN LIPOSOMES + SN
RAJI
CONTROL
a
b
c
% CD56+/CD69+ CELLS
% CD3+/CD69+ CELLS
% CD56+/CD69+CELLS
0 20 40 60 80
0 1 2 3 4
0 1 2 3 4 16
TIME (h)

Fig.7.

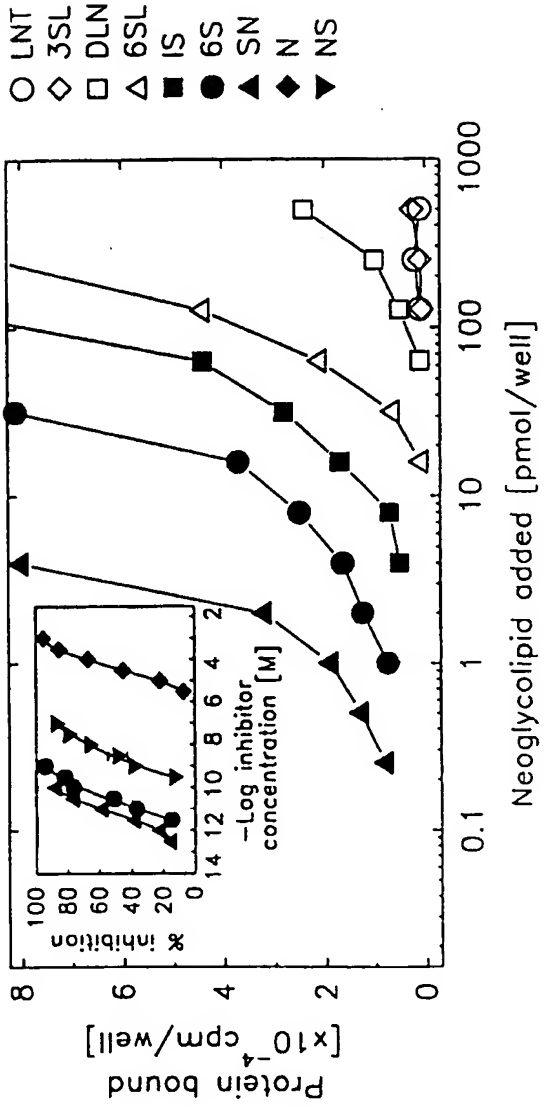
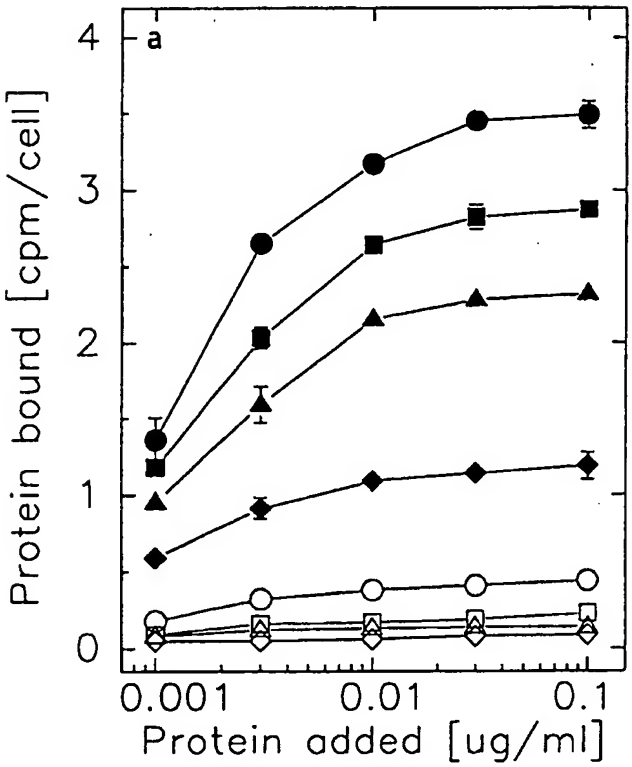

LNT
3SL
DLN
6SL
IS
6S
SN
N
NS
Protein bound
[x10⁻⁴ cpm/well]
Neoglycolipid added [pmol/well]
0.1
1
10
100
1000
0
2
4
6
8
% inhibition
100
80
60
40
20
0
-Log inhibitor concentration [M]
14 12 10 8 6 4 2

Fig.8. 11/14


a
Protein bound [cpm/cell]
0
1
2
3
4
0.001
0.01
0.1
Protein added [ug/ml]

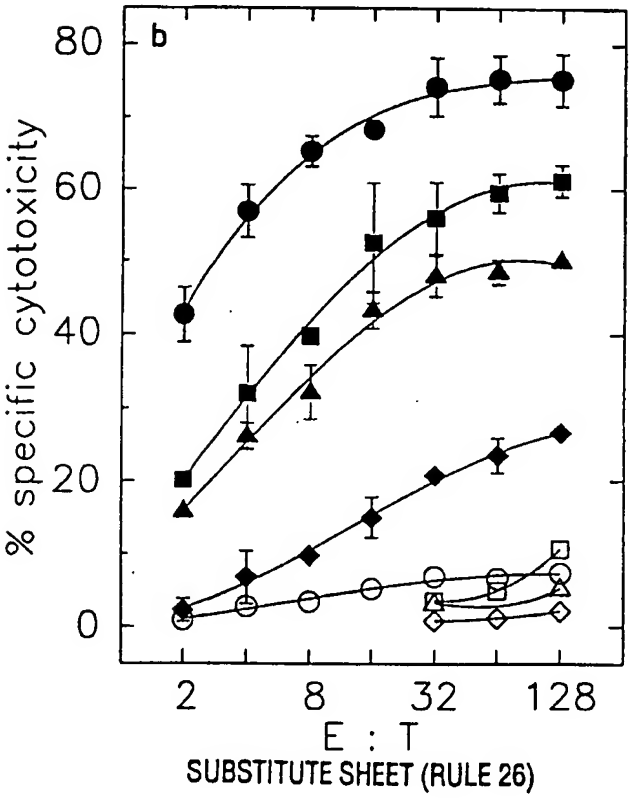
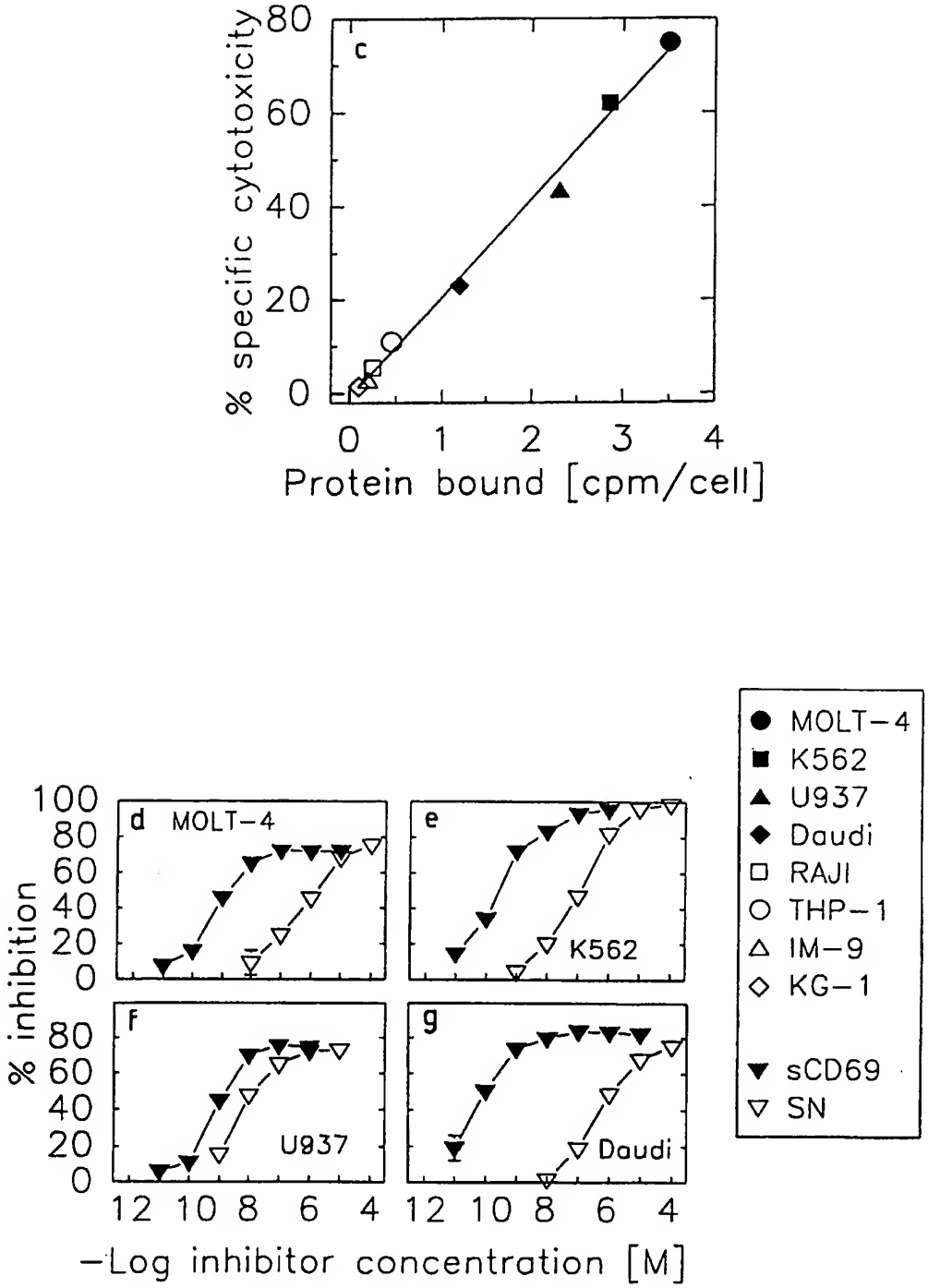

b
% specific cytotoxicity
0
20
40
60
80
2
8
32
128
E : T

Fig.8.


% specific cytotoxicity
80
60
40
20
0
c
0 1 2 3 4
Protein bound [cpm/cell]
% inhibition
100
80
60
40
20
0
d MOLT-4
e
K562
f
U937
g
Daudi
12 10 8 6 4 12 10 8 6 4
−Log inhibitor concentration [M]
MOLT-4
K562
U937
Daudi
RAJI
THP-1
IM-9
KG-1
sCD69
SN

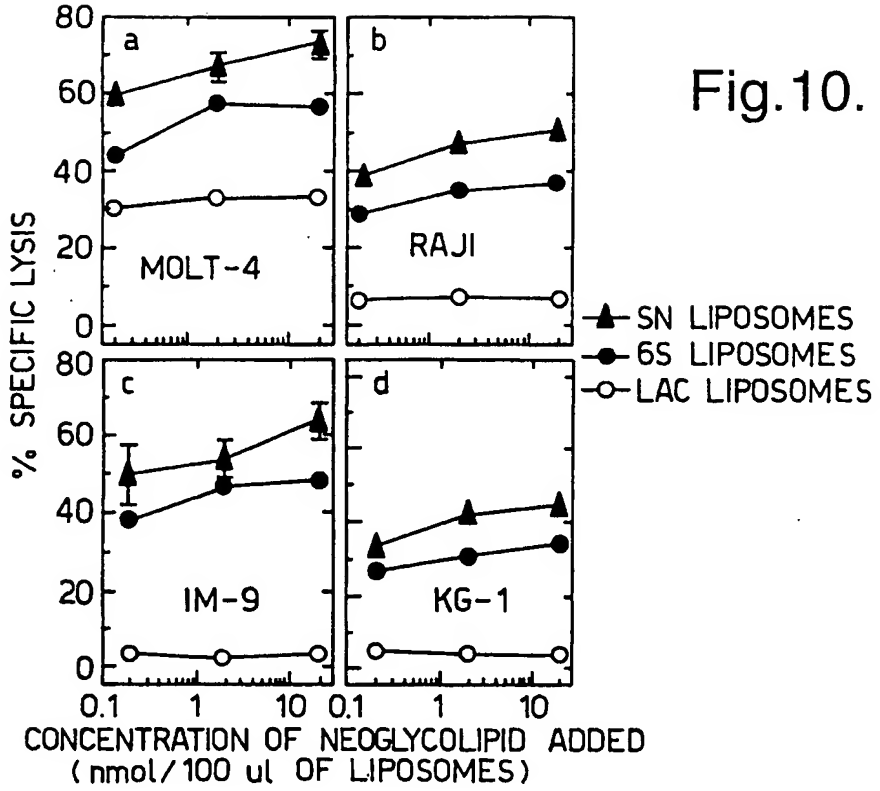

a
b
c
d
MOLT-4
RAJI
IM-9
KG-1
% SPECIFIC LYSIS
0
20
40
60
80
0.1
1
10
CONCENTRATION OF NEOGLYCOLIPID ADDED
(nmol/100 ul OF LIPOSOMES)
SN LIPOSOMES
6S LIPOSOMES
LAC LIPOSOMES

Fig.10.

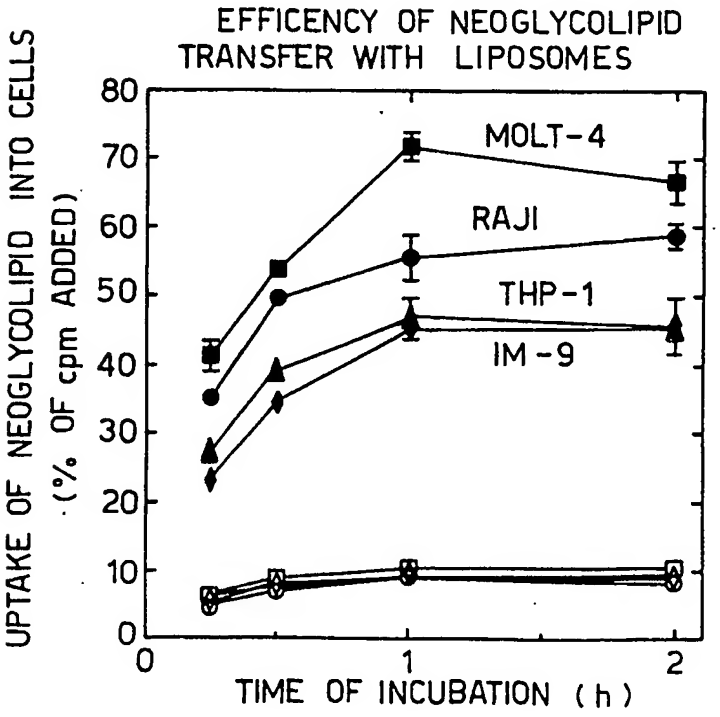
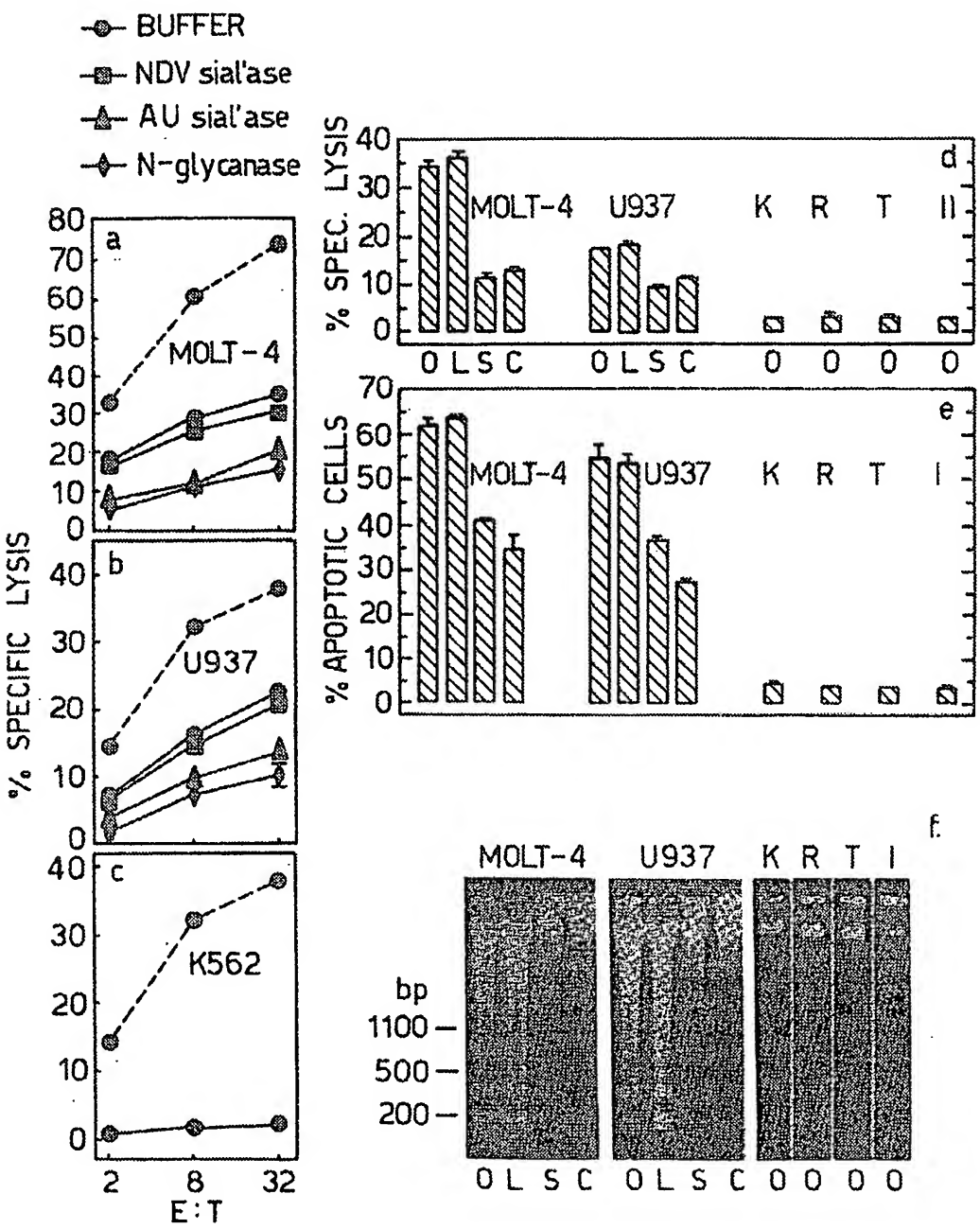

EFFICENCY OF NEOGLYCOLIPID
TRANSFER WITH LIPOSOMES
UPTAKE OF NEOGLYCOLIPID INTO CELLS
(% OF cpm ADDED)
0
10
20
30
40
50
60
70
80
MOLT-4
RAJI
THP-1
IM-9
0
1
2
TIME OF INCUBATION (h)

Fig.11.

Fig.12.


BUFFER
NDV sial'ase
AU sial'ase
N-glycanase
% SPECIFIC LYSIS
a
MOLT-4
b
U937
c
K562
E:T
2
8
32
% SPEC. LYSIS
d
MOLT-4
U937
K R T I
O L S C
% APOPTOTIC CELLS
e
f
bp
1100
500
200
O L S C O L S C O O O O

INTERNATIONAL SEARCH REPORT

Inter: nal Application No
PCT/GB 95/00321

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/70 A61K31/725 A61K31/715

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 208,no. 1, 8 March 1995 pages 68-74, BEZOUSKA, K. ET AL 'CD69 Antigen of Human Lymphocytes is a Calcium-dependent Carbohydrate-binding Protein' see the whole document ---	1-82
P,X	NATURE, vol. 372,no. 6502, 10 November 1994 page 150-157 BEZOUSKA, K. ET AL 'Oligosaccharide Ligands for NKR-P1 Protein Activate NK Cells and Cytotoxicity' see the whole document --- -/--	1-82

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 June 1995	28.06.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Mair, J

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INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/GB 95/00321

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269,no. 24, June 1994 pages 16945-16952, BEZOUSKA, K. ET AL 'Rat Natural Killer Antigen, NKR-P1, related to C-Type Animal Lectins is a carbohydrate-binding Protein' cited in the application see the whole document ---	71-82
P,X	NATURAL IMMUNITY, vol. 13,no. 4, 25 May 1994 page 191 BEZOUSKA, K. ET AL 'Rat NKR-P1 is a Carbohydrate-binding Protein' see the whole document ---	1-82
A	LECTINS: BIOLOGY, BIOCHEMISTRY, CLINICAL BIOCHEMISTRY, vol. 4, 1985 pages 354-367, BEZOUSKA, K. ET AL 'Binding of Serum Glycoprotein Asialooligosaccharides to the Porcine Liver and Leucocyte Membrane Lectins' see the whole document ---	1-82
X	LECTINS- BIOLOGY, BIOCHEMISTRY, CLINICAL BIOCHEMISTRY, vol. 6, 1988 pages 145-156, POSPISIL, M. ET AL 'Lymphocyte Membrane Lectins Functionally Important in NK Cytotoxicity' see the whole document ---	1-3,5,7, 16, 32-35, 37,39, 41,43, 52,68-82
X	LECTINS: BIOLOGY, BIOCHEMISTRY, CLINICAL BIOCHEMISTRY, vol. 7, 1990 pages 207-215, BEZOUSKA, K. ET AL 'Identification of Porcine Lymphocyte Membrane Lectins as Possible NK Cell Receptors Using New Derivatives of N-Linked GLycoproteins' see the whole document ---	1-3,5,7, 16, 32-35, 37,39, 41,43, 52,68-82
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, vol. 77,no. 5, May 1980 pages 2895-2898, STUTMAN, O. ET AL 'Natural Cytotoxic Cells Against Solid Tumors in Mice: Blocking of Cytoxicity by D-Mannose' see the whole document --- -/--	1,2,7, 16, 32-35, 37-39, 43,52, 68-82

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IMMUNOBIOLOGY, vol. 158,no. 3, 1981 pages 191-206, MUCHMORE, A.V. ET AL 'Spontaneous Cytotoxicity by Human Peripheral Blood Monocytes: Inhibition by Monosaccharides and Oligosaccharides' see the whole document ---	1-3,7, 16, 32-35, 37-39, 43,52, 68-82
X	IMMUNOLOGY LETTERS, vol. 12,no. 2-3, 1986 pages 83-90, POSPISIL, M. ET AL 'Lactosamine Type Asialooligosaccharide Recognition in NK Cytotoxicity' see the whole document ---	1-8,16, 32-35, 37,39, 41,43, 52,68-82
X	INTERNATIONAL JOURNAL OF CANCER, vol. 40,no. 1, July 1987 pages 12-17, ANDO, I. ET AL 'Ganglioside GM2 on the K562 Cell Line is recognized as a Target Structure by Human Natural Killer Cells' cited in the application see the whole document ---	1,3,5,7, 16,20, 32-35, 37,39, 41,43, 52,68-82
X	EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 213,no. 3, 1993 pages 1303-1313, BEZOUSKA, K. ET AL 'Characterization of the High-Affinity Oligosaccharide-binding site of the 205-kDa Porcine Large Granular Lymphocyte Lectin, a Member of the Leukocyte Common Antigen Family' see the whole document ---	1,3,5,7, 16,20, 32-35, 37,41, 43,52, 68-82
X	FOLIA MICROBIOL., vol. 38,no. 5, 1993 pages 421-431, KUBRYCHT, J. ET AL 'Peripheral Membrane Molecules of Leukocytes and NK Cytotoxicity' see the whole document ---	1,3,5,7, 16,20, 32-35, 37,41, 43,52, 68-82
A	SCANDINAVIAN JOURNAL OF IMMUNOLOGY, vol. 25, 1987 pages 507-515, ROELLINGER, S. ET AL 'Selective Inhibition of Human natural Killing and Antibody-Dependent Cellular Cytotoxicity by a Polyanion' cited in the application see the whole document --- -/--	1-82

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 95/00321

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 178,no. 2, August 1993 pages 537-547, LOPEZ-CABRERA, M. ET AL 'Molecular Cloning, Expression and Chromosomal Localization of the Human Earliest Lymphocyte Activation Antigen AIM/CD69, a New Member of the C-type Animal Lectin Superfamily of Signal-transmitting Receptors' see the whole document especially page 545, column 1 line 22-column 2, line 5 & column 2, line 19-27 ---	1-82
A	GLYCOBIOLOGY, vol. 1,no. 4, 1991 pages 321-328, MCCOY, J.P. ET AL 'Carbohydrates in the Functions of Natural Killer Cells' see the whole document especially page 325 -----	1-82

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Ir national application No.
PCT/GB95/00321

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-36 are directed towards a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.

2. [X] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims searched incompletely: 1-82
In view of the large number of compounds which are theoretically defined by the formula of claim 13 the search had to be restricted on economic grounds to the exemplified compounds and the general concept of the application.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

B2

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant *(for all designated States except US)*: **ALBOR BIOLOGICS, INC.** [US/US]; 569 Canton Street, Westwood, MA 02090 (US).

(72) Inventors; and

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(74) Agent: **ELRIFI, Ivor, R.**; Mintz, Levin, Cohn, Ferris, Glovsky, and Popeo, P.C., One Financial Center, Boston, MA 02111 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US (patent), UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
5 July 2007

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: IMMUNE REGULATION BASED ON THE TARGETING OF EARLY ACTIVATION MOLECULES

(57) Abstract: Disclosed are methods of treating subjects having disorders or conditions characterized by an unwanted immune response including administering an effective amount of an early activation molecule agonist, antagonist or depletor, to the subject. Human monoclonal antibodies specific to the early activation molecules, and methods of use, are also disclosed.

WO 2004/069183 A3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US04/02864

A. CLASSIFICATION OF SUBJECT MATTER
IPC: A61K 39/395(2006.01)

USPC: 424/133.1,144.1,181.1,183.1;435/810
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/133.1,144.1,181.1,183.1;435/810

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P	US 2003/0118592 A1 (LEDBETTER et al) 26 June 2003, pages 2-4, paragraphs [0011]-[0019], pages 4-5, paragraphs [0021]-[0029], page 14, paragraph [0105] and claims 17 and 35.	56-108
Y	MCINNES et al., Immunol Today. 1998 Feb;19(2):75-9, see entire document, in particular Box 1, Figure 2 and page 78 "therapeutic implications".	56-108
Y	EICHLER W, Differentially induced expression of C-type lectins in activated lymphocytes. J Cell Biochem Suppl. 2001;Suppl 36:201-8, see entire document.	56-108
Y	MCINNES et al., Interleukin-15 mediates T cell-dependent regulation of tumor necrosis factor-alpha production in rheumatoid arthritis. Nat Med. 1997 Feb;3(2):189-95, see entire document.	56-108

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
28 March 2007 (28.03.2007)	20 APR 2007
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized officer Zachary Skalding Telephone No. 571-272-9033

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US04/02864

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	SANCHO et al. CD69 downregulates autoimmune reactivity through active transforming growth factor-beta production in collagen-induced arthritis. J Clin Invest. 2003 Sep;112(6):872-82.	1-108

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US04/02864

Continuation of B. FIELDS SEARCHED Item 3:
MEDLINE, CAPLUS, EMBASE, BIOSIS, EAST
search terms: cd69, aicl, llt1, aim/cd69, rheumatoid, gastointestinal, respiratory, cardiovascular, alleg$, radioiso$, radioact$, $toxin$6, chemother$, sanchez-madrid